Cellular stress in the epidermis

Adapt or die!

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Contents

Abbreviations used in this Thesis

CK(s)	cytokeratin(s)
hARP	human acidic ribophosphoprotein
KGM	keratinocyte growth medium
KGM/-GF	keratinocyte growth medium depleted of growth factors
KGM/FCS	keratinocyte growth medium supplemented with 5 % foetal calf serum
ODN	oligodeoxynucleotide(s)
PO	phosphodiester
PS	phosphorothioate
PSPO	chimeric phosphorothioate/phosphodiester
SKALP	skin-derived antileukoproteinase
SLPI	secretory leukocyte proteinase inhibitor
TGase	transglutaminase
PMN	polymorphonuclear leukocyte
SOD	superoxide dismutase
UVB	Ultraviolet B light
MED	Minimal Erythema Dose
MAPK(s)	Mitogen Activated Protein Kinase(s)
NFκB	Nuclear Factor kappa B
TUNEL	TdT mediated dUTP Nick End Labelling
ERK	extracellular-signal-regulated kinase
JNK	c-Jun N-terminal Kinase
IL-#	Interleukin#
SSCP	Single Strand Conformation Polymorphism
TNF-α	Tumour Necrosis Factor alpha
IFN-γ	Interferon gamma
AP-1	Activator Protein 1
TN-C	tenascin-C
FCS	Foetal Calf Serum

General introduction

1. Skin

1.1. Skin, a flexible and protective tissue.

Skin is a unique tissue in many ways. It forms the last barrier from the inside out and the first barrier from the outside in. It is this barrier function that forces the skin, and especially the epidermis, to meet high criteria in both physical and biological properties. It regulates the body temperature and protects the skin from water loss. It protects the body from the damaging effects of penetrating chemicals or radiation. And in the case of loss of barrier function, for instance by wounding, it closes the ruptured barrier as quickly as possible and in the meantime forms a first line of defence against micro-organisms and viruses that have crossed the epidermal barrier before wound closing. The skin has to form a reliable protective barrier under normal conditions and must be capable of quick tissue regeneration and initial host protection if necessary. The physiological reliability and flexibility that is described above is the result of a highly co-ordinated process of cellular proliferation and differentiation that can be adapted to changing circumstances and that is accurately regulated in a very complex manner. All these properties make the regulation of keratinocyte growth and differentiation a very interesting subject for study from a biological point of view. Moreover information on the possibility to influence the regulatory mechanisms could ultimately lead to new therapeutic targets in the treatment of epithelial disorders concerning epidermal growth and differentiation.

1.2. The structure of skin

Human skin consists of an outer epithelial layer, the epidermis, and an inner connective tissue layer, the dermis. These two layers are physically separated by a basement membrane. The dermis assumes the important functions of thermoregulation and supports the vascular network to supply the avascular epidermis with nutrients. The dermis contains mostly fibroblasts that are responsible for secreting collagen, elastin and proteoglycans that give the support and elasticity of the skin. Also present are immune cells that are involved in defence against foreign invaders passing through the epidermis [1,2].

The epidermis is the outermost layer of the skin and provides the first barrier of protection. The epidermis consists mainly of cells called keratinocytes. The epidermis is subdivided into four layers or *strata*, the *stratum basale* (SB), the *stratum spinosum* (SS), the *stratum granulosum* (SGR) and the *stratum corneum* (SC) through which a keratinocyte gradually migrates from the dermal epidermal junction to the surface. The *stratum basale* (SB (also called *stratum germinativum*)) provides the germinal cells necessary for the regeneration of the layers of the epidermis. After a mitotic division a newly formed cell is pushed toward the surface and a process of terminal differentiation begins as the cell migrates to the surface. The cells that divide in the *stratum germinativum* soon begin to accumulate many desmosomes on their outer surface which provide the characteristic 'prickles' of the *stratum spinosum* (SS), which is often called the prickle-cell layer. The progressive maturation of a keratinocyte is characterised by the accumulation of keratin,

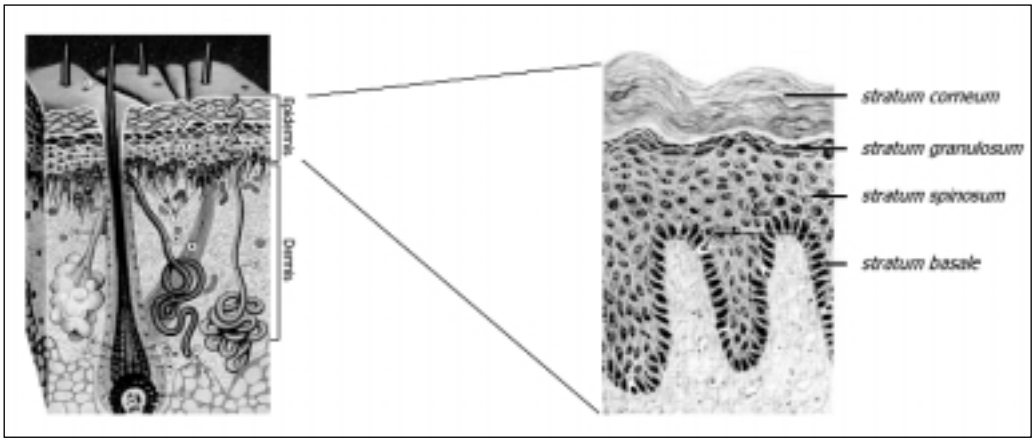

Figure 1. The structure of human skin consisting of the dermis covered by the epidermis. In the magnification of the epidermis the different layers of cells *(strata)* that are formed by the terminal differentiation of the keratinocytes are clearly visible and indicated.

called keratinisation. The cells of the *stratum granulosum* (SGR) accumulate dense basophilic keratohyalin granules. These granules contain lipids, which along with the desmosomal connections, help to form a waterproof barrier that functions to prevent fluid loss from the body [1,2].

As a cell accumulates keratohyalin granules, it is thought that rupture of lysosomal membranes release lysosomal enzymes that eventually cause cell death. The dead and dying cells filled with mature keratin form the *stratum corneum* (SC). The deeper cells of the stratum corneum retain their desmosomal junctions, but as they are pushed to the surface by newly forming cells of the *stratum basale*, the dead cells gradually break apart and are lost, a process called desquamation [1,2].

2. Epidermal growth and differentiation

2.1. Epidermal differentiation

The keratinocytes in the *stratum corneum* (corneocytes) have lost their nuclei and other recognisable cell organelles and comprise 65% insoluble cysteine-rich fibrous proteins called cytokeratins (CKs). There is a very characteristic expression pattern of CKs in the epidermis. The basal cells are characterised by the expression of CK5 and CK14. As the differentiating keratinocytes migrate upward in the epidermis they start to express the cytokeratins CK1 and CK10. The expression of these CKs (CK1 and CK10) is specific for normal epidermal differentiation. In the stratum granulosum, release of filaggrin from the keratohyalin granules induces the aggregation of CKs forming macrofibers. In addition

epidermal differentiation involves the synthesis of a highly insoluble cornified envelope whose cross-linking is catalysed by Transglutaminases 1 and 2 using the precursor protein involucrin as a first scaffold. In addition to formation of keratin macrofilaments and the cornified envelope, changes also occur during terminal differentiation in the synthesis of intercellular lipids, membrane glycoproteins and intercellular adhesion molecules (integrins). This complex terminal differentiation process leads to the continuous formation and renewal of a tight barrier layer [1,2].

In situations of hostile environmental changes, like e.g. loss of barrier function or epidermal damage due to genotoxic compounds or ionising radiation, the epidermal keratinocytes are capable of switching to a second pathway of differentiation [3,4,5]. This pathway of differentiation takes place in the context of hyperproliferation where almost all basal keratinocytes are mitotic. This epidermal phenotype is generally referred to as regenerative maturation [6,7], also called the activated keratinocyte phenotype [8,9]. In a number of epidermal pathological conditions, like e.g. psoriasis [10] and skin tumours [11], this keratinocyte phenotype can also be found where it has no obvious physiological function and in most cases is directly responsible for the outer characteristics of many skin diseases. The alternative differentiation pathway is characterised by the upregulation of the expression of proteins that are normally involved in the formation of the corneocyte like involucrin and transglutaminase. The most obvious difference and main characteristic of the regenerative maturation phenotype, is the *de novo* synthesis of a number of proteins that are absent in normal skin but are highly expressed during this pathway of regenerative maturation. Among these proteins are the cytokeratins CK6, CK16 and CK17 [3,12,13] and the proteinase inhibitor SKALP [14,15] (which is an acronym for SKin derived Anti-Leuko-Proteinase). The expression of these proteins is tightly associated with the epidermal phenotype of regenerative differentiation. Although it is not likely that any of these proteins have an actual function in the initiation or regulation of the differentiation switch itself, these proteins can form the key to unravelling the mechanisms underlying the transition between the different epidermal differentiation pathways. It is our belief that any information on the regulatory pathways, regulatory (co)factors that are involved in, and the environmental conditions that are prerequisite for the initiation and regulation of the expression of these proteins, should be informative on the regulation of the total phenotype. Knowledge on the regulatory pathways underlying the epidermal phenotypes may lead to tools that can be used for targeted manipulation of these phenotypes. Ultimately this ability can provide us with new therapeutic targets and tools for the treatment of skin disorders where the activated skin phenotype is responsible for cosmetic and clinical problems.

2.2. Epidermal proliferation

With respect to their proliferative characteristics tissues can be divided in three groups. In nerve and skeletal muscle there is no cell division. In other tissues like e.g. liver cell division can occur in response to injury, a process called conditional renewal. And finally in tissues like skin and mucosa (stratified squamous epithelia) and the gastrointestinal tract

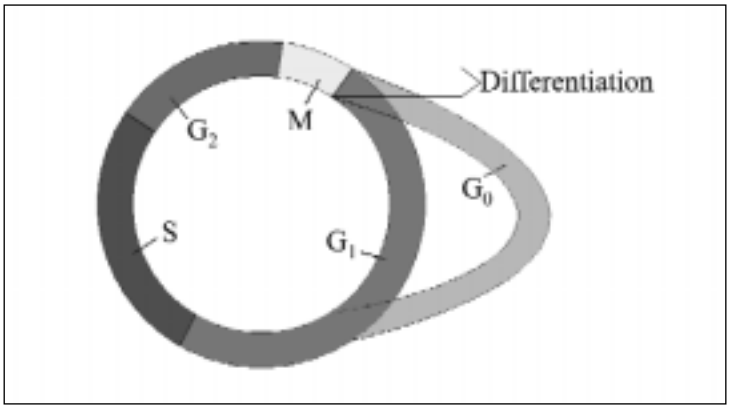

Figure 2. Schematic representation of the cell cycle. Indicated are the different phases of this cycle (G0/G1/S/G2/M). After the actual mitosis the cell can continue in the G1 phase, become quiescent in G0 or start to differentiate. From G0 the cell can reënter the cell cycle in G1 at a given time.

(simple epithelia) there is a permanently renewing population of differentiating cells with a very short life span originating from a relatively small number of dividing 'stem' cells. Skin is a classical example of a tissue with these kind of proliferative characteristics. The rate of cell shedding at the surface of this tissue must exactly match the rate of cell division in the basal layer in order to maintain normal skin thickness, although disturbed ratios of cell division and cell loss occur in pathological states.

The basis of cell proliferation is the cell cycle. The cell cycle can be divided into four separate stages, G1- (and G0-), S-, G2- and M-phase. The G1 (Gap 1) phase is characterised by gene expression and protein synthesis. This gap phase enables the cell to grow, to produce and gather all the necessary proteins and co-factors needed for the next phase and to replicate cell organelles. The cell may pause at the end of its G1 phase for some time before initiating DNA synthesis in the S phase. This time is known as the restriction or start point. If the cell remains at this point for a long period of time without replicating its DNA, it is said to be in the G0 phase. The S (Synthesis) phase is characterised by the replication of the cell's DNA. After DNA replication is completed the cell enters the third cell cycle stage, the G2 (Gap 2) phase. During the G2 phase the cell prepares itself for the actual division by e.g. synthesising necessary proteins and condensing the chromosomal DNA. Finally the cell enters the fourth and final phase of the cell cycle: the M (Mitosis) phase. During the M phase the actual cell division takes place. This division can be separated into two separate events, the division of the cell's chromosomes (the actual mitosis, also called karyokinesis) and the division of the cell's cytoplasm in two daughter cells (called cytokinesis). At this stage the cell cycle is completed and the cell has two choices, either to enter the cycle again or become a resting cell in G0 (called cell quiescence). At this stage of the cell cycle the cell can also choose to escape the cell cycle and start to terminally differentiate (see figure 2). In normal skin a basal keratinocyte divides about once every 13 days spending the majority of its time in the G1/G0 phase of the cell cycle. Maturation/differentiation and shedding of a newly formed keratinocyte lasts about 26 days. In contrast, the cell cycle of a psoriatic keratinocyte is shortened to about 1.5 days and the maturation/differentiation and shedding phase to about 4 days [1,16].

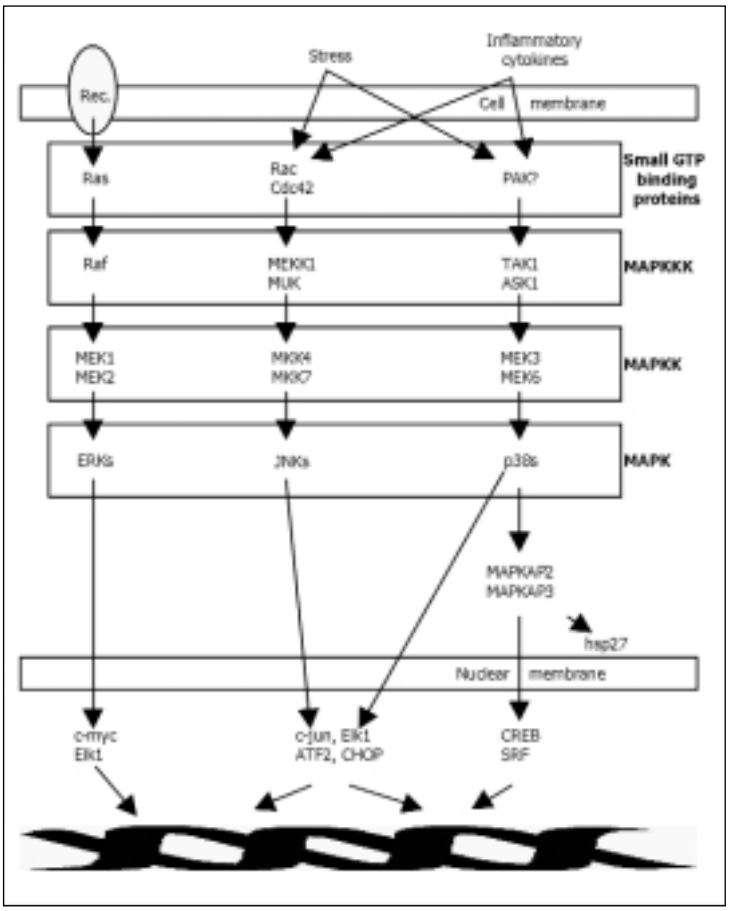

Figure 3. Schematical overview of the eukaryotic stress response pathway leading to the activation of the Mitogen Activated Protein Kinases (MAPKs). Through different stages of protein phosphorylation extracellular signals like (pro)inflammatory cytokines, growth factors or cellular stress are transduced through the cell, finally leading to the alteration of the cell's gene expression profile through activation of transcription factors in the nucleus.

2.3. Apoptosis

Apoptosis or programmed cell death is a physiological form of cell death that plays a pivotal role in tissue (re)formation during embryogenesis and metamorphosis but also occurs in adult tissue during regenerative processes. Cell death occurs throughout the life span of the organism and represents the ultimate differentiative decision made by cells. It is characterised by specific morphological changes such as DNA fragmentation, membrane blebbing, cellular shrinkage and disassembly in membrane-enclosed vesicles (apoptotic bodies). Many hormones, cytokines and growth factors are known to act as general and/or tissue-specific survival factors preventing the onset of apoptosis. In addition, many hormones and growth factors are also capable of inducing or facilitating programmed cell death under physiological or pathological conditions, or both (for reviews see [17,18]). Apoptosis has become a hot topic of research during the last few years. Insight into the process of cell death will not only contribute to the understanding of basic developmen-

tal issues, but will also facilitate the development of therapeutic interventions that could alter the course of disease. Since all cells have the apoptosis machinery required to commit suicide encoded in their genome, the ability to initiate it in a lineage-specific, non-inflammatory manner would allow for instance the irradiation of specific (skin)cancers. Alternatively, inhibition of cell death pathways could rescue valuable but condemned cells, such as HIV infected CD4+ T cells or dopaminergic neurons in Parkinson's disease.

Caspases are members of a family of cysteine proteases that orchestrate the intracellular biochemical events that enable cells to kill themselves by apoptosis. Caspases are highly specific and efficient proteases with an unusual and absolute requirement for cleavage after aspartic acid [19], a specificity shared only by the cytotoxic lymphocyte serine proteinase Granzyme B. A tetrapeptide sequence and additional tertiary structures are involved in substrate recognition. Activated caspases cleave a restricted set of proteins rather than causing indiscriminate protein digestion. The affected proteins are usually cleaved, in a coordinated manner, at one site only resulting in loss or change of function. The overall picture of how caspases lead the apoptotic process is not fully understood. From a number of structural and regulatory proteins it is known that they undergo endoproteolytic cleavage by various caspases. An example of the proteolysis of a regulatory protein is the cleavage of the inhibitor of 'caspase activated deoxyribonuclease' (CAD) called ICAD by caspase-3. In non-apoptotic cells CAD is held inactive in the cytoplasm by ICAD. During apoptosis ICAD is inactivated, leaving CAD free to function as a nuclease leading to DNA fragmentation [20,21]. An example of a structural element that is a target for caspase cleavage are the lamins, the structural component of the nuclear lamina. During apoptosis lamins are cleaved at a single site by caspases, causing the nuclear lamina to collapse. Other known caspase substrate examples are the cleavage of Bcl-2 proteins , protein kinase C delta [24-26], the retinoblastoma protein [27,28] and a number of proteins involved in RNA splicing [29], cytoskeletal organisation [30,31] and DNA repair [32]. Although the exact involvement of these kinds of cleavages in the process of cell death is not completely clear it is obvious that the cellular homeostasis is disabled and that cellular disassembly is facilitated.

The caspase cascade can generally be activated in two different ways, either through receptor ligand interactions at the surface of the predestined cell or by intracellular signals. These intracellular signals involve cytochrome c release from mitochondria. In the cytosol, cytochrome c combines with APAF-1 (Apoptotic Protease Activating Factor) in the presence of ATP to activate caspase-9 that, in turn, activates effector caspases such as caspase-3. Bcl-2 and related proteins control cytochrome c release from the mitochondria. Plasma membrane receptors such as Fas (CD95, APO-1), characterised by a so-called 'death domain' in their cytoplasmic part, can activate the caspase cascade after ligand binding through adaptator molecules such as FADD (Fas-Associated protein with a Death Domain). This route leads to the activation of caspase 8 and subsequently to the activation of the effector caspases.

With respect to skin there are some parallels between the normal terminal differentiation pathway of keratinocytes in the epidermis and the process of programmed cell death, apoptosis. In both cases the cells are initiated to die in an organised fashion and in both cases there is an orderly removal of the cells nuclei and organelles. Although in nor-

mal epidermis only few apoptotic cells (as determined by TUNEL-staining) can be detected, there have been reports that at least a part of the general apoptosis machinery, i.e. members of the caspase family, is activated during the normal terminal differentiation process in keratinocytes [33]. The activated caspases are thought to play a possible role in the normal loss of the keratinocytes nuclei at the end of the differentiation process, perhaps together with proteins involved in the formation of the cornified envelope [34]. Disturbance of this process may lead to parakeratosis that is seen in many pathological skin conditions [35]. It should however be emphasised that terminal differentiation and apoptosis are obviously two distinct processes, one leading to the formation of a wanted cell and the other leading to the removal of an unwanted cell.

In skin, apoptosis plays also a very important role in reacting to environmental changes resulting in cellular stress. UV light is the major extrinsic factor affecting the keratinocyte and it is well established that UV exposure can lead to premature skin ageing (photoageing) and has carcinogenic effects. UV irradiation damages proteins, lipids, DNA [36] and leads to the activation of signal transduction pathways involved in the cellular stress response. Depending on the nature and the quantity of cellular damage, the cell either decides to repair damage, survive and react or adapt to the environmental changes or, when cellular damage is too large, decides to sacrifice itself by undergoing apoptosis. This pathway of UV induced epidermal apoptosis leads to the formation of the well-known 'sunburn' cells that are TUNEL positive. In this manner apoptosis plays a role in the prevention of malignant cell formation. There are however examples in which apoptosis itself is directly the cause of a pathological skin condition like e.g. in the case of 'toxic epidermal necrolysis' (TEN), a Fas ligand mediated massive onset of epidermal apoptosis induced by the use of certain drugs .

3. Regulation of epidermal growth and differentiation

Cellular proliferation and differentiation in the epidermis are fine-tuned in order to maintain epidermal integrity and epidermal homeostasis. Because the epidermis is subjected to a variety of environmental hazards, this integrity and homeostasis is constantly being threatened. The epidermis possesses a broad array of regulatory systems to respond to these threats. Through signalling molecules, receptor ligand interactions and cytoplasmic changes, intracellular signal transduction pathways are activated. These pathways are responsible for the amplification and mediation of the initial signal to the nucleus. In the nucleus, the cell can change its transcription factor repertoire and, by doing so, change its transcription pattern. In that way the cell can respond to extracellular influences by either adapting to the 'new' environment through the synthesis of new proteins that can act in cellular defence strategies and adapt the proliferation and differentiation patterns of the cell or, in case of severe cell damage, the cell can go into apoptosis. In the next few paragraphs I will give a brief overview of the regulatory mechanisms that are known to exist in keratinocytes and the regulatory factors that are involved in these routes.

3.1 Regulation through extracellular factors

3.1.1 Extracellular factors regulating epidermal proliferation

Regulation of stem cell proliferation is an important topic since the rate of stem cell proliferation determines the rate at which the differentiating cells enter the upper epidermal layers. Many factors have been identified that influence the proliferative rate of keratinocytes and most of these factors also have effects on keratinocyte differentiation as well. Most of the studies on these factors have been done in *in vitro* model systems and the actual situation in skin is still not completely understood. Growth factors like Epidermal Growth Factor (EGF), transforming Growth Factor alpha (TGF-α) (both sharing the EGF receptor) and Insulin like Growth Factor (IGF) have shown to enhance both keratinocyte proliferation [39,40] and migration [41,42]. In psoriatic skin the IGF and the EGF system is altered compared to the situation in normal epidermis, there is an overexpression of EGF-receptors (EGFR) [43] and IGF-receptors (IGFR) [44]. Treatment with antipsoriatic agents downregulate the number of IGFR [44] and EGFR [43] in keratinocytes. In addition it has been shown that EGF is directly involved in regulating the expression of CK6 and CK16 [45,46] in hyperproliferative keratinocytes, suggesting a possible role for these two signalling complexes in the psoriatic keratinocyte phenotype. Another growth factor relevant for keratinocyte proliferation is Keratinocyte growth factor (KGF). KGF is like EGF, TGF-α and IGF a potent epithelial cell mitogen. However, studies indicate that in contrast to EGF, KGF plays a role in inducing normal keratinocyte differentiation [47]. The increase in the expression of KGF and its receptor (KGFR) in psoriatic skin correlates with the pool of proliferative keratinocytes in the affected epidermis of prosiasis patients. This observation together with the fact that the expression of the KGFR is downregulated upon treatment with ultraviolet-B light (UVB, an established anti-psoriatic treatment) suggests a role of the KGF pathway in the typical hyperproliferative keratinocytes in the basal and lower suprabasal layers in the psoriatic epidermis [48].

Besides growth factors also inflammation mediators, like Interleukin-1 (IL-1 [49,50]), Interleukin-4 (IL-4 [51]) and Interleukin-6 (IL-6 [52,53]) display stimulatory effects on keratinocyte proliferation. There have also been reports on other signalling molecules that stimulate keratinocyte proliferation like nitric oxide (NO [54]), Granulocyte/macrophage colony stimulating factor (GM-CSF [55,56]) and Substance P (SP [57]). In contrast to these factors that specifically induce or stimulate keratinocyte proliferation there are factors that have opposite effects and suppress or block proliferation in keratinocytes. The most extensively studied factor is Transforming Growth Factor beta (TGF-β). Many studies have shown the repression of keratinocyte proliferation by TGF-β *in vitro*. The presumed mechanism involves the regulation of the retinoblastoma gene product (pRB) function by TGF-β [58,59]. This is supported by the fact that events that compromise the function of the pRB protein (e.g. the presence of viral proteins with pRB binding domains) uncouple TGF-β regulation of proliferation [60]. There are several indications that other factors that have repressive effects on keratinocyte proliferation as well as on other cell types, like e.g. tumour necrosis factor alpha (TNF-α [61,62]), interferon gamma (IFN-γ [61]) and 1,25-dihydroxyvitamine D3 [63] also mediate these effects through pRB [63-66]. The tumour

suppressor gene product pRB seems to play a central role in the repression of keratinocyte proliferation and there are indications that proliferation repression by a number of factors through pRB could be a mechanism for the initiation of keratinocyte terminal differentiation [67-70]. The studies mentioned here, in which factors were identified that influence keratinocyte proliferation, were done in a variety of different model systems. It is therefore unclear whether these effects coexist, whether they interact meaningfully and what their relative roles are.

3.1.2 Extracellular factors regulating epidermal differentiation

As indicated in the previous paragraph it is very difficult to uncouple the regulation of keratinocyte differentiation from the regulation of proliferation. The cessation of keratinocyte proliferation is a prerequisite for the initiation of normal terminal differentiation. It is therefore obvious that factors that repress keratinocyte proliferation could be the first signal for terminal differentiation whereas factors that stimulate proliferation indirectly prohibit the initiation of differentiation. In contrast a recent study from Pariamo *et. al* describes the opposite event, where ectopic expression of proteins like CK10 and CK16 that are normally exclusively expressed by differentiating cells drastically influence cellular proliferation [71]. It is likely that there are many factors that influence both the proliferation and differentiation in such a way that it eventually tips the 'cellular scale' in favour of differentiation and the cell dedicates itself to this terminal maturation route. Factors that act in such way, both on repression of proliferation and the induction of differentiation are IFN-γ [72,73] and 1,25-dihydroxvitamin D3 [75].

A very extensively studied inducer of keratinocyte differentiation is calcium. In normal epidermis there is a gradient of calcium. Calcium concentrations in the outer, more differentiated layers are higher than in the more basaly located epidermal cell layers [76]. This suggested role for calcium in the keratinocyte differentiation process was confirmed by several *in vitro* studies. These studies showed that in the presence of low levels of calcium (<0.1 mM), low expression levels of differentiation markers could be found. However when the concentration of calcium in the culture medium was elevated to 1.5-2 mM, differentiation is markedly increased as measured by the expression of marker proteins [77-79]. Recent reports describe a calcium receptor on the keratinocyte that can be expressed in two different forms due to alternative splicing. The shortest form lacks a part of the extracellular domain and is inactive. Speculations are made that the ratio of expression of these two receptors on a cell can determine the calcium sensitivity of that cell and thereby regulate the differentiation response to calcium [80,81]. Another very important mechanism by which differentiation is regulated is through contact with the basement membrane and extracellular matrix. Several studies have indicated that loss of integrin mediated attachment of keratinocytes to several proteins in the basement membrane, leads to cell-cycle withdrawal and commits the cells irreversibly to terminal differentiation [82-86]. In addition also the composition of the extracellular matrix can determine the cell's fate with respect to terminal differentiation. Studies have shown that exposure of differentiating keratinocytes to fibronectin leads to inhibition of differentiation specific gene expression [82] and sustained keratinocyte proliferation [87].

3.2. Regulation through signal transduction routes

3.2.1. Signal transduction routes in skin

Intracellular signal transduction pathways are the tools by which cells can link events that take place at the level of the plasma membrane to downstream events that take place at the cytoplasmic or nuclear level. After initial receptor-ligand binding or cytosolic changes, intracellular protein cascades conduct the signal through the cell to the nucleus where transcription factors are activated. Through the alteration of the cell's transcription factor repertoire, appropriately altered gene expression is established. These signal transduction pathways are responsible for the transmission, amplification and diversification of the initial signal and are therefore fundamental to the life and death of cells.

As in other cell types, many signal transduction routes are known to be functional in keratinocytes. These signal transduction routes can roughly be divided in a few distinct groups with respect to their substrate specificity. The protein tyrosine kinases like the EGF receptor, the IGF receptor, the Focal Adhesion Kinase (FAK) and the Janus kinases (JAKs) have been described in keratinocytes. As mentioned earlier the EGF-receptor and the IGF-receptor but also the FAK are involved in regulating keratinocyte proliferation and migration [39-42,88]. The JAKs are well known for their role in the activation of a group of transcription factors called the Signal Transducers and Activators of Transcription (STATs) [89,90] that is known to be involved in the regulation of CK17 expression by keratinocytes [91].

A second group of protein kinases are the serine/threonine kinases like amongst many others, the protein kinase C (PKC), the cyclic AMP dependent protein kinase A (PKA) and the Mitogen Activated Protein Kinases (MAPKs). PKC has been reported to be involved in repression of keratinocyte proliferation [92,93] and regulation of the expression of late keratinocyte differentiation markers induced by calcium [94,95]. The role of PKA in skin remains obscure but is linked with cAMP dependent regulations of keratinocyte proliferation and differentiation [96]. The MAPKs is a group of kinases that is activated upon cellular stress and proinflammatory cytokines and will be described in more detail later.

Another group of signal transduction molecules is the phospolipases. The phospholipases are enzymes that hydrolyse various bonds in the head group of phospholipids and are important for the degradation of damaged or aged cell membranes. Phospholipase A2 is involved in the release of arachidonic acid from membranes and its subsequent conversion into leukotrienes and prostaglandins. This route has shown to be deregulated in hyperproliferative skin and prostaglandin E2 induces proliferation in keratinocytes [97,98]. A second phospholipase called phospholipase D (PLD) catalyses the hydrolysis of membrane phospholipids to produce phosphatidic acid which can further be converted into diacylglycerol (DAG) that can act as a second messenger. A third phospholipase is phospholipase C (PLC), which function in keratinocytes is unclear. However an isoform of PLC with a substrate specificity for sphingomyelin called sphingomyelinase (SMase) has been implicated in a great number of regulatory processes in keratinocytes. SMase can hydrolyse spingomyelin into ceramide and phosphorylcholine after which ceramide can serve as a second messenger. Ceramides could be involved in the regulation of keratinocyte differentiation, proliferation and apoptosis [99-101]. With respect to changes in differentiation

phenotypes induced by extracellular signals like wounding, UV-B irradiation, epidermal water loss and inflammation, the MAPK signal transduction pathway is extremely important. For that reason I will describe the MAPK routes into further detail in the next few paragraphs.

3.2.2. The Mitogen Activated Protein Kinase cascade (MAPK)

One of the best-studied signal transduction pathways is the Mitogen Activated Protein Kinase cascade (the MAPK cascade). A MAP kinase cascade is universally composed of three protein kinases acting in series. From the nucleus up: a MAP kinase (MAPK) that is activated upon phosphorylation by a MAPK kinase (MAPKK) which in turn is activated upon phosphorylation by a MAPKK kinase (MAPKKK). These MAPKKKs are activated through interaction with small GTP binding proteins that belong to the Ras superfamily. The MAPKs can carry the information to the nucleus by virtue of their ability to undergo nuclear translocation upon activation. The MAPKs have two main characteristics. First they are activated by dual phosphorylation of a Threonine and a Tyrosine amino acid residue. Second they are proline directed Serine/Threonine protein kinases with a minimum consensus target of *Ser/Thr-Pro*. The MAPKs consists of three main family members, the extracellular regulated kinases (ERKs), the c-jun N-terminal kinases (JNKs) and p38 kinases each of which is activated by its own MAPKK and further upstream cascade. For a schematical overview see figure 3.

3.2.1. The extracellular regulated MAP kinases (ERKs)

The activation of the ERKs is by far the best understood mammalian MAP kinase pathway and leads to the activation of the ERK family members ERK1 (p44) and/or ERK2 (p42) [102,103]. The MAPKKS MEK1 or MEK2 activates the MAPKs ERK1/ERK2. MEK1 and MEK2 can activate both ERK1 and ERK2; there seems to be no strict substrate specificity. In their turn the MAPKKs MEK1 and MEK2 are activated by c-Raf, a MAPKKK that is activated by the GTP bound form of Ras. This Ras activity is initiated through the activation of receptor tyrosine kinases like for instance the EGF receptor or the PDGF receptor which are two well established mechanisms for the activation of ERK1 and ERK2 [104]. Obviously these receptor tyrosine kinases can become active upon binding of the ligand to its receptor but there are also ligand-independent mechanisms known. For example the EGF receptor that can become active through voltage dependent calcium channels resulting in activation of Ras leading to increased ERK kinase activity [105]. A second mechanism of calcium dependent Ras activation is mediated by the calcium sensitive Ras guanine nucleotide-release factor (Ras-GRF) that is expressed in brain neurons [106]. Once activated the ERKs can phosphorylate a number of cytoplasmic proteins including phospholipase A2 [107] and interestingly c-Raf-1 and MEK1 suggesting that they may regulate their own signalling pathway [108]. Activated ERK1 and ERK2 have the ability to translocate to the nucleus where they can phosphorylate and thereby activate a number of transcription factors including c-myc [109,110], Elk-1 [111,112] and STAT proteins [113]. In skin an involvement of the ERKs in signalling processes was shown in melanocytes [114] and dermal fibroblasts [115]. In human keratinocytes it is well known that exposure to UVB

leads to the activation of the ERKs through phosphorylation of the EGF receptor [116,117]. There is increasing evidence that this UV induced ERK activity plays a role in the erythema and oedema seen after UV irradiation through the biosynthesis of prostaglandin [118]. In addition to these effects of UV irradiation on ERK activation, it was demonstrated that cell adhesion could also activate the ERKs. Integrin dependent cell adhesion leads to the activation of a Focal Adhesion Kinase (FAK) and ultimately activates Ras leading to the activation of ERK1 and ERK2 [119,120]. This can have important implications for cell growth and differentiation by the extracellular matrix with respect to keratinocyte or fibroblast migration in processes like wound healing and re-epithelialisation.

3.2.2. The c-jun N-terminal MAP kinases

The c-Jun N-terminal Kinases (JNKs, also known as Stress Activated Protein Kinases (SAPKs)) is a MAP kinase family group that was first identified as a kinase activity that bound and phosphorylated the transcription factor c-jun on Ser-73 and Ser-63 within its NH_2-terminal activation domain [121]. Subsequently several human JNK isoforms have been cloned and are known as JNK1, JNK2 and JNK3. The transcripts of each of these genes are alternatively spliced to create mRNAs that encode 46 kDa and 55 kDa JNK isoforms. These JNKs are activated upon dual phosphorylation by the MAPKKs MKK4 and MKK7, whereas MKK4 can activate both the MAP kinases p38 and JNK, MKK7 can exclusively activate JNKs. The MAPK kinases MKK4 and MKK7 can become active upon phosphorylation by the MAPKK kinases, MEKK1 [122,123], MUK [123] and Tpl-2 [124]. These MAPKK kinases are initially activated by GTP binding proteins of the Rho family (e.g. Rac-1 and Cdc42 [125,126]). The JNKs are activated by exposure of cells to environmental stress (ionising radiation, heat shock, oxidative stress, osmotic shock) or by treatment with pro-inflammatory cytokines. Targets of the JNKs include the transcription factors c-jun [127], Elk-1 [128] and ATF-2 [129] (Activating Transcription Factor 2). The transcription factors c-jun and ATF-2 both belong to a group of proteins that can form AP1 complexes as homo- or heterodimers with other proteins. The JNKs are therefore the major regulatory factor in the formation of active AP1 complexes in mammalian cells in response to cellular stress. In keratinocytes JNK activity is induced upon UV-B irradiation and photodynamic therapy [130-132] resulting ultimately in elevated levels of AP-1. These elevated levels of AP-1 have been implicated in the induction of abnormal epidermal gene expression and photoageing [133,134].

3.2.3. The p38 MAP kinases

Finally the third group of MAPKs is the p38 family consisting of five members i.e. p38α, p38β_1, p38β_2, p38γ and p38δ [135-137]. The p38s are the mammalian homologues of the yeast stress activated protein kinase HOG1 [138] and were initially identified in mammalian cells as a kinase that was activated by hyperosmolarity and endotoxic (LPS) shock [139], and was essential for cytokine production by monocytes [140]. Additional studies have shown that p38 MAP kinases can be activated by a number of other environmental stimuli as well, e.g. UV radiation and proinflammatory cytokines [141-143]. Two p38 MAP kinase activating kinases have been cloned, i.e. the MAPKKs MKK3 and MKK6 [144,145]. The MAPKK MKK4

is also capable of activating p38 as well as JNK MAP kinases. Although there have been several reports on potential MAPKKKs that activate MKK3 or MKK6, like e.g. MLK3 [146], MTK1 [147], ASK1 [148] and TAK1 [149] it remains largely unclear how this activation is mediated *in vivo*. Upon activation, p38 MAP kinases can activate a number of substrates. It has been shown that p38 can in its turn activate two kinases termed Mitogen Activated Protein Kinase Activated Protein Kinase 2 and 3 (MAPKAP-K2 and MAPKAP-K3) [150,151]. The physiological substrates of these MAPKAP kinases are small heat shock protein 27 [152,153] and transcription factors like CREB (cAMP response element binding protein) and the serum response factor (SRF) [154]. A recent report describes the involvement of MAPKAP-K2 in the transport of activated p38 from the nucleus to the cytosol as a regulator of cellular localisation of p38 [155]. Other downstream substrates for p38 MAP kinases are transcription factors like CHOP [156], ATF-2 and Elk-1 [157]. In keratinocytes p38 is activated upon UV irradiation [141,158] and is implicated in the regulation of matrix metalloproteinase expression [159,160].

3.3 Regulation through transcription factors

Many keratinocyte specific genes discovered so far were found to be regulated at the transcriptional level. The fact that many of these genes are expressed in a co-ordinated and stratum-specific manner might predict a common, stratum-specific expression regulation mechanism. Studies done so far did not reveal such a common regulation pattern at the transcriptional level but a rather individual strategy where genes are individually regulated by at least one regulatory agent. In addition, comparison between the promoter regions of epidermal genes did not reveal large regions of high homology. Transcription factors regulating epidermal genes tend to be of the 'generic' type and it is possible that epidermal or stratum specific gene expression is achieved through the action of a network of factors with low individual epithelial specificity. Nevertheless the intensive analysis of epidermal gene regulation has resulted in the identification of factors regulating the various differentiated epidermal states.

3.3.1. Transcription regulation in the basal layer

Keratins 5 and 14 are expressed in the basal layer of most stratified epithelia including the epidermis. A number of studies have identified a set of transcription factors that may be important in CK5 gene regulation including Activator Protein 1 (AP1), Activator Protein 2 (AP2) and Sp1 [161,162]. The promoter region of CK5 seems to consist of two major regions, one responsible for differentiation specific gene expression and the other for tissue specific gene expression [163]. In the promoter of the bovine CK5 gene, an AP1 site seems to confer tissue specific CK5 expression, however footprinting analysis of this region in CK5 expressing and non-expressing cells produced identical results [164]. The promoter of acidic CK14, the molecular partner of CK5, also contains a consensus element for AP2, this sequence element is important for gene activity but is not sufficient for tissue specific gene expression [165].

In addition to general transcription factors like AP1, AP2 and Sp1, there are also

transcription factors that are mainly used by keratinocytes. Basonuclin is an example of a transcription factor originally identified in basal keratinocytes and thought to be keratinocyte specific, but it has been detected in other cell types as well and is correlated with proliferative activity [166]. Studies have indicated that keratinocyte differentiation is preceded by the removal of basonuclin from the nucleus to the cytoplasm [166,167]. In addition it has been shown that the transcription factor NFκB has a repressive effect on the promoter activity of both the K5 and the K14 promoter [159] and, moreover has growth inhibitory effects [168]. NFκB family includes proteins p65 (RelA), p50, p52 and c/rel, which homo- and heterodimerise amongst themselves [169-171]. Activation of these proteins is not dependent on new protein synthesis but they are stored in the cytoplasm where they are held inactive bound to an inhibitory protein, IκB. It is known that NFκB complexes can interact with other transcription factors like C/EBP and AP1 and regulate transcription These data suggest that removal of basonuclin and activation of NFκB could be part of the transition from basal gene expression and cell proliferation towards terminal differentiation.

3.3.2. Transcription regulation in normally differentiating keratinocytes

Calcium, an element capable of inducing keratinocyte differentiation can directly stimulate the expression of several differentiation related genes such as CK1 [174], loricrin [79], involucrin [78,175] and transglutaminase [175]. The 3' end of the CK1 gene contains a region, comprising an AP1 site and two hormone response elements that regulates this calcium responsiveness [176]. The induction of differentiation by calcium is accompanied by an increase in AP1 activity in the nuclei of keratinocytes [177], and many genes associated with terminal differentiation possess functional AP1 consensus sites in their promoter regions [178] including involucrin [179-181], transglutaminase [182], CK1 [183] and loricrin [184,185]. However it must be taken into account that there are indications that these AP1 consensus sites might play a role both in activation and repression of gene activity, depending on the composition of the AP1 transcription factor complex. AP1 transcription factor complexes function as homo- or heterodimers of jun (c-jun, junB or junD), fos (Fra-1, Fra-2, c-fos or fosB) and Activating Transcription Factor (ATF). The actual effect of AP1 binding to its consensus sequence might be dependent on the composition of the AP1 complex [186,187,178]. This could also play a role in differential stimulation of genes by AP1 in the basal and suprabasal layers of the epidermis. In addition to AP1 also other general transcription factors have been found to play a role in keratinocyte or differentiation specific regulation of gene expression. In addition to AP1, transcription factors like AP2, Sp1 and POU domain proteins also play a dominant role in the transcription regulation of many genes expressed during the differentiation process of the keratinocyte including involucrin [188], transglutaminase-1 [189] and transglutaminase-3 [190]. Whereas it was mentioned that NFκB was thought to play a role in the induction of epidermal differentiation, there are no studies that identify NFκB as a regulator of differentiation markers. However several studies in mice in which the NFκB pathway was disrupted indicate that lack of NFκB activity results in loss of epidermal homeostasis indicating its importance in maintaining this homeostatic situation [191-193].

3.3.3. Transcription regulation in activated keratinocytes

Environmental signals such as growth factors, cytokines and irritants can modulate the activation of nuclear transcription factors, thus regulating gene expression. This regulation is very important in skin where a large variety of signals appear, especially in disease states or in wound healing, to stimulate new gene expression in keratinocytes. Several studies have indicated that the expressed genes that are characteristic for activated keratinocytes [12], i.e. CK6, CK16 and CK17 are regulated at the transcriptional level by distinct regulatory mechanisms [194]. As during normal differentiation, transcription factor complexes like AP1, AP2 and Sp1, are also involved in the regulation of gene expression in activated keratinocytes [13,194,195]. However there are regulatory processes that are restricted to the activated keratinocyte phenotype as well. An example is the regulation of CK17, a keratin associated with several skin diseases, skin tumours and skin irritation [196,-198]. The expression of the CK17 gene can be induced by the exposure of keratinocytes to IFN-γ and this expression is regulated at the transcriptional level by the activation and translocation of the transcription factor STAT1/91 (Signal Transducer and Activator of Transcription) to the nucleus of the keratinocytes. Through binding of STAT-1 to Gamma Interferon Activation Sites (GAS) in the CK17 promoter, CK17 gene expression is induced [91,199-201]. Another transcription factor that is known to be activated by cellular stress like inflammation and radiation is NFκB. The study by Ma *et al* that was mentioned two paragraphs ago showed that in addition to a repressive effect on the CK5 and CK14 promoter, the CK6 promoter is strongly activated by NFκB [159]. However studies using bandshift or promoter deletion mutant never confirmed the regulatory role of NFκB in abnormal epidermal gene expression.

4. Models for studying epidermal growth and differentiation

In the past years many models for the investigation of epidermal growth and differentiation have been described and used. Being on the outside of every organism, skin is one of the few organs that can be studied *in vivo* in a non-invasive manner. In addition one can easily obtain biopsies that can be studied biochemically, immunohistochemmically or that can be used for further cell biological characterisation. In the next two paragraphs a number of *in vivo* and *in vitro* models that are commonly used for the analysis of both normal and abnormal epidermal differentiation will be described.

4.1 In vivo models for studying epidermal growth and differentiation

Obviously a great advantage of *in vivo* models in comparison with keratinocyte monocultures is the fact that it closely resembles the *in situ* situation. Especially interactions between the different cell types in skin and the interaction of skin cells with the organism's immune system are important advantages of *in vivo* models. Data obtained from these models are bound to be relevant for the actual situation in normal skin, in contrast to data from *in vitro* models that should be interpreted with more caution with respect to the actual *in situ* situation.

A well-known standardised human model for epidermal injury is the repeated application of adhesive tape to the skin in order to remove the stratum corneum called 'tape stripping'. This tape stripping results in hyperproliferation of the affected skin accompanied by inflammation and abnormal differentiation [202-204]. Other models are the application of detergents and other skin irritants to normal skin [205,206], the study of the spreading margin of a psoriatic plaque [207-209], controlled wounding of skin [210,211] and UV irradiation of normal human skin [212,213]. From an ethical point of view these human models are limited in their application with respect to experimental procedures. Therefore in addition to these human models, animal counterparts for every model mentioned above are also known and are used for epidermal research [214-216]. The disadvantage of these animal models is the fact that the skin of most animals is quite different from human skin with respect to thickness and anatomy. For that reason a number of xenotransplantation models have been developed in which normal or pathological skin is transplanted on (mostly) mice [217-220]. There are however disadvantages on all these in *vivo models*. They do not form an unlimited source of material and they can not be exactly controlled. This has been one of the reasons for the development of a number of *in vitro* models for keratinocyte growth and differentiation.

4.2 In vitro models for studying epidermal growth and differentiation

Scientists have been culturing keratinocytes for over 100 years. In 1897 C.A. Ljunggren reported that excised pieces of skin remained viable when cultured in ascites fluid at room temperature, and that the skin could subsequently be grafted onto wounds [221]. During the first half of the twentieth century, as *in vitro* culture techniques were developed, experiments were carried out on explants of skin and the epidermal sheets that migrated out from them (for review see [222]). Later cultures were established from desegregated keratinocytes, but high seeding densities were required and subculture was rarely successful (reviewed in [223]). A major breakthrough came in 1975, when Rheinwald and Green reported that epidermal keratinocytes could be cultured at clonal density if maintained in the presence of a feeder layer of lethally irradiated 3T3 mouse embryo cells [224]. Large numbers of cells can be grown from small biopsies; it has many applications and is still widely used. In the years after the introduction of the method by Rheinwald and Green a lot of work has been done to obtain a more defined growth medium for keratinocytes. The first step was to develop culture conditions with the ability to grow keratinocytes in the absence of feeder cells or conditioned medium [225,226]. And the ultimate aim was to develop defined culture media for keratinocytes without the addition of serum or other crude protein extracts (like e.g. bovine brain extract) [227,228]. Since a few years these serum free media are commercially available.

In our own department we have developed a number of keratinocyte culture models in which keratinocyte differentiation can be manipulated. Depending on the composition of the culture medium the keratinocytes differentiate either normally or follow the regenerative maturation pathway [229,77]. These models are very useful in the analysis of epidermal differentiation switches. In addition to cultures of primary keratinocytes one can also use

immortalised keratinocytes. The great advantage of these immortalised cells is that they can be passaged indefinitely in contrast to primary keratinocytes. The most commonly used immortalised keratinocyte cell line is HaCaT [230], a spontaneously immortalised cell line, but keratinocytes can also be immortalised by viruses [231]. These monolayered cell culture systems of human skin cells have several advantages. However submerged monolayered cultures also have some inherent limitations. For instance, only compounds soluble in culture medium can be tested and due to the absence of a stratum corneum many properties of the skin are altered. To overcome these limitations many research groups are developing artificial skin cultures for both research and therapeutic purposes. In these culture models keratinocytes are cultured on matrices containing fibroblasts or on dermis obtained from human cadaver skin [232-234]. One of the main advantages of these three-dimensional skin cultures is that it their biological and physical properties highly resemble the actual *in vivo* situation. Moreover cell-cell, cell-matrix and dermal-epidermal interactions can be studied in these models. In addition to these skin equivalent cultures also skin explants can be used. These skin explants are normally fresh skin biopsies that are maintained in culture for a few days [235,236].

5. SKALP expression as a paradigm for abnormal epidermal maturation

As mentioned earlier Skin Derived AntiLeukoProteinase is a proteinase inhibitor that was in initially found in, and purified from the scales of psoriasis patients [237,238] and is also known as elafin [239] or ESI [240] (elastase specific inhibitor). The gene encoding SKALP is approximately 1.7 kb long and contains 3 exons and 2 introns. The first exon encodes the hydrophobic signal peptide of the protein with a length of 22 aminoacids and the first 4 aminoacids of the mature protein. The second exon encodes the rest of the mature protein and the third exon is non-coding. The 5' non-coding region of the gene contains both a putative TATA and CCAAT box, located respectively 94 and 120 bases upstream of the translation start site. Cleavage of the signal peptide results in a mature protein with a molecular mass of 9.9 kDa comprising two separate protein domains with respect to structure and function. The N-terminal end of the mature protein consists of a region containing multiple functional transglutaminase (TGase) crosslinking consensus sequences [241] (consensus: GQDPVK), suggesting that the protein can be crosslinked into the cornified envelope in the maturing corneocyte. The C-terminal domain of the protein, harbouring the proteinase-inhibiting domain of SKALP, contains 8 cysteine residues that can form 4 disulphide bonds that stabilise this domain. This structure is called a *4 disulphide core* structure also called a *whey acidic protein* motif (WAP motif/domain). SKALP shares homology with a number of proteins based upon the first domain (TGase substrate domain), with a number of proteins based upon the second domain (proteinase inhibiting domain) and with protein based upon both the domains. Proteins homologous to SKALP with respect to the TGase are members of the REST family (Rapidly Evolving Seminal vesicle Transcribed) [242,243] that also share homology with SKALP in non-coding regions of the gene. A protein that shares homology with SKALP based upon the second protein

domain (WAP motif and proteinase inhibiting domain) is SPLI also known as ALP. There are many other proteins that contain a WAP motif like e.g. whey proteins, adhesion molecules and scorpion toxins. And finally a group of proteins that was recently identified as a new protein family share homology with SKALP based upon both domains in the protein. These proteins were recently extensively reviewed and assigned the family name TRAPPINs (stands for TRansglutaminase substrate and wAP domain containing ProteINs (SKALP now also referred to as trappin-2)), referring to its property of getting 'trapped' in a tissue and thereby functioning as an anchored protein [244,241]. These proteins are thought to have evolved from REST genes and WAP domain containing proteins by means of exon shuffling.

SKALP is not expressed in normal human epidermis but is induced and highly expressed under inflammatory skin conditions (e.g. psoriasis), in skin tumours and after wounding [245,246]. In general it can be said that SKALP gene expression is induced in differentiating keratinocytes that follow the regenerative maturation/differentiation pathway.

6. Aim and Outline of this thesis.

General aim

The switch in epidermal differentiation pathways/phenotypes from the normal epidermal differentiation pathway to an activated state of differentiation (known as regenerative maturation/differentiation), is found in response to a number of environmental stimuli. These stimuli include inflammatory skin conditions (e.g. psoriasis), wound healing, osmotic stress (tape stripping) and UV-B irradiation. Very little is known about the regulatory mechanisms that are at the basis of these induced changes in human epidermal differentiation. The general aim of this thesis was to gain more insight into the regulation of these switches in epidermal differentiation using SKALP as a read-out. Because SKALP expression is very tightly associated with the phenotype of regenerative differentiation it is our working hypothesis that knowledge about the pathways, factors and mechanisms that regulate SKALP expression will be informative on the regulation of the complete phenotype. We have used SKALP induction and expression as a paradigm for the induction and expression of the epidermal phenotype of regenerative maturation/differentiation in order to identify extracellular factors, intracellular signal transduction pathways and transcription factors involved in the onset of this epidermal phenotype. We speculate that knowledge of these pathways will contribute to our understanding of physiological responses of skin to injury (e.g. wound healing) or pathological conditions such as psoriasis.

Specific aims

1. Because SKALP was used as a model protein throughout our studies we first wished to characterise this molecule extensively at the protein and genetic level. Chapter 2.1 addresses the question of SKALP expression *in vivo* (tissue localisation, subcellular localisation, constitutive versus inducible expression), and we investigated its potential function in tissue homeostatis. In chapter 2.2 we have investigated the possibility that genetic abnormalities in SKALP expression could be responsible for the pathology associated with pustular forms of psoriasis.

2. Using cell culture models recently developed in our lab we have wanted to identify extracellular factors (cytokines, growth factors) that could induce SKALP gene expression in cultured human keratinocytes. Chapter 3.1 shows that out of a large array of stimuli tested, only TNF-α and serum were potent inducers of SKALP expression at the protein and mRNA level.

3. Once the extracellular factors that could induce SKALP expression were identified, we wanted to elucidate the signalling pathways that were involved in relaying the external signals (serum, TNF) to the nucleus a potential inducer. We have investigated the involvement of MAP kinase signal transduction routes in the induction of SKALP gene expression and found that p38 activity is crucial for the induction of SKALP gene expression by FCS and TNF-α in cultured human keratinocytes, as described in chapter 3.1. When the work on SKALP expression by keratinocytes was in progress, we had identified the extracellular matrix molecule tenascin-C as another gene that was expressed by keratinocytes under conditions of external stress. We therefore wanted to investigate if expression of tenascin-C and SKALP were subject to similar regulatory mechanisms. We therefore applied the same culture models to study the regulation of tenascin-C for comparison with SKALP. Chapter 3.2 shows that p38 activity is not crucial for the expression of tenascin-C, which can be induced both by IL-4 and TNF-α.

4. Once the main inducers for SKALP (serum, TNF) and a potential signalling pathway were identified (p38 MAPkinase) in vitro we wanted to know if these factors were relevant for regulation of expression in vivo. Therefore we developed and characterised an *in vivo* model for the induction of SKALP expression in normal human skin. Chapter 3.3 shows that UV-B irradiation of normal skin leads to the expression of SKALP, and is preceded by activation of p38 and JNK. *In vitro*, JNK and p38 are also activated upon UV-B irradiation but this is not sufficient to induce expression of SKALP. A remarkable finding in these experiments was that UV-B induced p38 activity appears to be important for cell survival following UV-B irradiation.

5. Because the in vitro cell culture models used in our studies were not ideally suited to study induction of SKALP (e.g. lack of SKALP induction by UVB in vitro as opposed to in vivo) we wanted to develop in vivo or ex vivo models to study regenerative differentiation and SKALP induction in intact human skin. We therefore used, and further characterised the nude (immunodeficient) mouse transplantation model. In this model we can induce/maintain and study both normal and regenerative epidermal differentiation with the same characteristics as normal human skin. Secondly we have used and characterised a

whole skin explant model system for the induction of abnormal differentiation. We demonstrate the usefulness of the latter model for studies using antisense oligonucleotides directed against SKALP.

6. Finally we wanted to study the transciptional regulation of SKALP by analysis of the promoter region of the SKALP gene. Chapter 4 describes the characterisation of the SKALP promoter region: determination of the transcription start site, cell-specific expression, deletion analysis of the promoter region using transient transfection of reporter constructs. No definite conclusions with regard to regulation by specific transcription factors could be made.

SKALP: Functional aspects and its putative role in disease processes

Constitutive and inducible expression of SKALP/Elafin provides anti-neutrophil elastase defense in human epithelia

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Abstract

Skin-derived antileukoproteinase (SKALP), also known as elafin, is a serine proteinase inhibitor first discovered in keratinocytes from hyperproliferative human epidermis. In addition to the proteinase inhibiting domain which is directed against polymorphonuclear leukocyte (PMN) derived enzymes such as elastase and proteinase 3, SKALP contains multiple transglutaminase (TGase) substrate domains which enable crosslinking to extracellular and cell envelope proteins. Here we show that SKALP is constitutively expressed in several epithelia that are continuously subjected to inflammatory stimuli, such as the oral cavity and the vagina where it co-localises with type 1 TGase. All epithelia from sterile body cavities are negative for SKALP. In general, stratified squamous epithelia are positive, whereas pseudostratified epithelia, simple/glandular epithelia and normal epidermis are negative. SKALP was found in foetal tissues of the oral cavity from 17 weeks gestation onwards where it continued to be expressed up to adult life. Remarkably, in foetal epidermis SKALP was found from week 28 onwards, but was downregulated to undetectable levels in neonatal skin within 3 months, suggesting a role during pregnancy in foeto-maternal interactions or in the early maturation phase of the epidermis. Immunoelectron microscopy revealed the presence of SKALP in secretory vesicles including the lamellar granules. In culture models for epidermal keratinocytes we found that expression of the endogenous SKALP gene provided protection against cell detachment caused by purified elastase or activated PMNs. Addition of exogenous recombinant SKALP fully protected the keratinocytes against PMN-dependent detachment whereas superoxide dismutase and catalase were only marginally effective. These findings strongly suggest that the constitutive expression of SKALP in squamous epithelia, and the inducible expression in epidermis participate in the control of epithelial integrity, by inhibiting PMN derived proteinases.

Introduction

Systemic regulation of proteinase activity is performed by numerous plasma-derived inhibitors that are involved in regulation of complement activation, clotting and fibrinolysis. In addition to these systemic antiproteinases a number of locally acting inhibitors is known. These include inhibitors of cysteine proteinases such as the cystatins [247], and tissue inhibitors of metalloproteinases [248]. Although the vast majority of the serine proteinase inhibitors are found in the circulation, a limited number of this class is locally active at the tissue level. Secretory leukocyte proteinase inhibitor (SLPI), an inhibitor of the polymorphonuclear leukocyte derived proteinases elastase and cathepsin G, is produced by cells of mucosal surfaces and is found in the corresponding epithelial lining fluid [249]. In previous studies we and others have described an epidermal proteinase inhibitor which was found to be partially homologous to SLPI. This inhibitor, termed Skin-derived antileukoproteinase (SKALP), otherwise known as elafin or ESI, is absent in normal epidermis but is highly expressed in psoriatic epidermis and in some epithelial cell lines [239,250-253]. SKALP inhibits

PMN-derived elastase and proteinase 3 and is therefore putatively involved in regulation of cutaneous inflammation [254,255]. We have previously characterized SKALP in biochemical and cell biological studies [246,251]. The cDNA and gene of SKALP have been cloned and sequenced, and the chromosomal localisation was assigned to chromosome region 20q12-q13 [251,256,257]. Apart from the C-terminal domain that harbours the proteinase inhibiting properties, the SKALP molecule also contains a domain with putative transglutaminase substrate motifs that enable crosslinking to the cornified envelope proteins [251,258]. Previous studies in epidermis, cultured keratinocytes, and human epidermal tumours showed that SKALP expression patterns appear to be similar to that of cytokeratin 16, which is known to be expressed in the context of hyperproliferation (see [259,260] for reviews on epidermal differentiation). We have recently obtained evidence that decreased levels of SKALP could be implicated in the pathogenesis of pustular forms of psoriasis [261]. Apart from its presence in inflamed epidermis, SKALP/elafin has been reported in tracheal tissue [262] and in bronchial secretions, although the exact cellular source of sputum-derived SKALP/elafin *in vivo* is unclear [252].

It is not known to what extent SKALP is expressed in normal adult human epithelia, and no functional studies have been performed so far. Here we report that SKALP is constitutively expressed in many adult epithelia that are exposed to environmental stimuli. In these tissues the presence of inflammatory cells is physiological, suggesting that SKALP provides protection against excessive proteolysis. Although normal human epidermis is negative, SKALP was transiently expressed during foetal and neonatal development of the epidermis. In functional assays using cultured cells we observed a protective effect of epithelial SKALP expression against the action of leukocytic proteinases, suggesting that SKALP is involved in the maintenance of epithelial integrity.

Materials and methods

Materials

Goat-anti-rabbit IgG biotin conjugate, avidin-alkaline phosphatase, and the chromogenic substrates 5-bromo-4-chloro-3-indolyl phosphate, nitroblue tetrazolium, Ca-ionophore 23187 and aminoethyl carbazole (AEC) were obtained from Sigma Chemicals, St. Louis, MO, USA. Swine-anti-rabbit Ig conjugated with horseradish peroxidase (SwarPO) was obtained from Dakopatts, Glostrup, Denmark. The Vectastain ABC kits for monoclonal and polyclonal antibodies purchased from Vector Laboratories Inc., Burlingame, CA, USA. A synthetic peptide comprising amino acid 23 to 36 of SKALP was synthesised by Eurosequence, Groningen, The Netherlands; at the C-terminus a cysteine residue was added for coupling to a carrier protein. The sulfo-SMCC kit and Sulfolink gel were obtained from Pierce, Rockford, IL, USA. RNA zol™ B was obtained from Cinna/Biotex Laboratories, Inc., Houston, TX, USA, and [α-^{32}P]-dCTP from Amersham, UK. Ficoll-paque was from Pharmacia, Uppsala, Sweden.

Recombinant SKALP was a kind gift from Dr. Norman Russell, ICI Pharmaceuticals, UK. As a probe for ribosomal 28S RNA a 2.1 kb EcoRI/BglII fragment was used (a kind gift of Dr Jan Bauman, TNO Rijswijk, The Netherlands).

Tissues
Archival and fresh biopsy/autopsy material was used for this study. The following adult tissues were studied with respect to SKALP expression: brain; cornea; tongue; palate/lingual tonsil; gingiva; pharynx; larynx; epiglottis; vocal fold; lung; bronchus; esophagus; duodenum; colon; liver; kidney; urethra; uterine cervix; vagina; skin from various regions including head, ear, breast, axillar region, mamilla, inguinal region, pubic area, scrotum, finger, and foot sole; and hair follicle. Punch biopsies from adult skin were taken from normal human volunteers and psoriatic patients. Foetal and neonatal tissues were available from: a 9 week old embryo which could be examined *in toto*, two second trimester foetuses, five third trimester foetuses and two neonates. Specimens used for immunohistochemistry were fixed in buffered 4% formalin for at least 24 h, and embedded in paraffin. Tissues for *in situ* hybridisation and northern blot analysis were snap frozen in liquid nitrogen and stored at -20° C.

Antisera
A polyclonal antiserum against recombinant SKALP was obtained as described previously [255]. An antiserum against a synthetic peptide comprising amino acids 23 to 36 of SKALP was prepared by coupling the peptide via a C-terminal cystein residue to chicken ovalbumin, using the sulfo-SMCC procedure according to the manufacturer's instructions. This conjugate was used for immunisation of a rabbit according to previously described protocol [238]. Control serum (preimmune serum) was drawn before the immunisation procedure. The antiserum against the synthetic peptide was affinity purified using the synthetic peptide coupled to Sulfolink coupling gel according to the manufacturer's instructions. The two antisera against recombinant SKALP and against the synthetic peptide gave identical staining patterns in histological sections. A monoclonal antiserum against type 1 transglutaminase was obtained from Sanbio, Uden, The Netherlands. A monoclonal antibody against human PMN elastase was obtained from DAKO, Denmark. Mon150, a monoclonal antibody against involucrin was obtained as previously described [263]. Ks8.12, a monoclonal antibody recognising CK 13 and 16 was from Sigma, St Louis MO, USA; a monoclonal antibody against CK 10 (DE-K10) was obtained from ICN, Costa Mesa.CA, USA.

Immunohistochemistry
Biopsies were fixed in buffered 4% formalin for at least 24 hours and processed for embedding in paraffin. Sections (6μm) were mounted on 3-aminopropyltriethoxy-silane (Sigma, St Louis MO, USA) coated slides. Sections were deparaffinised and rehydrated and used for an indirect peroxidase technique. For antigen retrieval from paraffin sections, the slides were pre-treated for 15 min in 0.1% trypsin/0.1% $CaCl_2$ pH 7.8 by 37°C for anti-cytokeratin 10, 30 min in 10mM citrate buffer for Ks8.12 and for Mon150 a pre-treatment of 10 min in 10mM citrate buffer using a microwave oven (Miele, M720) at 450 Watt was performed. After preincubation with 20% normal swine or rabbit serum the slides were incubated for 60 min with the antibodies and after washing with phosphate-buffered saline (PBS) they were incubated with peroxidase-conjugated swine-anti rabbit Ig or rabbit-anti-mouse Ig for 30 min. A solution of AEC in sodium-acetate buffer pH 4.9 containing 0.01% H_2O_2 was added for 15 min after preincubation with sodium-acetate buffer

pH 4.9. When desirable, the slides were counterstained with Mayer's haematoxylin (Sigma, St Louis, MO, U.S.A.) and mounted in glycerol gelatine. Appropriate controls with pre-immune sera or omission of the primary antibodies were performed.

Immunocytochemical staining of human keratinocytes cultured on coverslips

Human keratinocytes were cultured on tissue culture coverslips (Thermanox, LABTEK Division, Miles Laboratories Inc., Naperville, IL, USA) using different culture conditions (see below). Cells were fixed using either paraformaldehyde (1%) or aceton/methanol (50/50). Fixed cells were stored at -20°C. Before usage the coverslips were transferred to 4°C for 30 min and then dried in air at room temperature. Paraformaldehyde fixed cells were incubated for 15 min in 50 mM NH_4Cl (in PBS, pH 7.5), and aceton/methanol fixed cells were incubated for 15 min in PBS. Cells were incubated for 10 min at 37°C with either 20% normal goat serum for the polyclonal antibodies (anti-SKALP) or 20% normal horse serum for the monoclonal anti-TGase antibody. After this incubation the coverslips were washed for 15 min in PBS and incubated with the primary antibody for 1 hour at 37°C. Coverslips were washed for 15 min in PBS and incubated for 30 min at 37°C with either goat-anti-rabbit-biotinylated antibody or horse-anti-mouse-biotinylated antibody in a dilution of 1:200. Following this incubation coverslips were washed and incubated with a complex of avidine and biotinylated horse radish peroxidase (1:50) for 30 min at roomtemperature. Finally, coverslips were washed for 15 min in PBS and incubated with diaminobenzidine tetrahydrochloride (DAB) substrate (metal enhanced DAB substrate Kit; Pierce, Rockford, Illinois, USA), and the reaction was terminated by incubating the coverslips in demineralised water. The coverslips were lightly stained with hematoxyline solution (Harris-type; Sigma Diagnostics, St. Louis, Missouri, USA) and embedded in histological mounting medium (Permount; Fisher Scientific, New Jersey, USA) before making photographs.

Construction of plasmids and synthesis of RNA probes

We have previously cloned the SKALP cDNA and gene [251,257]. From the cDNA a 251 bp PCR product was obtained (bp +69 to +320, relative to the translation start site), which was subcloned into a pGEM-4 T-vector (Promega, Madison, Wi, USA) in both orientations. The plasmid was linearised with *Pst*1 to synthesise the antisense cRNA, or in the reverse orientation the control sense cRNA by T7 polymerase, using a digoxigenin RNA labelling kit (Boehringer Mannheim, Germany). The cRNA's were fragmented by limited alkaline hydrolysis and used for in *situ* hybridisation.

In situ hybridisation

Frozen sections (10μm) were placed on superfrost slides (Menzel-Glazer) and heated for 2 min at 50°C. The sections were fixed in phosphate-buffered saline (PBS) containing 4% (v/v) paraformaldehyde for 10 min at room temperature, air-dried and rehydrated in 2x SSC (standard sodium citrate) 2x 5min. Sections were then prehybridized in hybridisation buffer for at least 1 hour, consisting of 4x SSC, 10% dextrane, 50% formamide, 0.5mg herring sperm DNA (Boehringer, Mannheim, Germany), 2mM ethylenediaminetetratic acid

(EDTA) and 1x Denhardt's solution. Prehybridisation and hybridisation was performed at 37°C. Prehybridisation fluid was drained off and the slides were washed in 2x SSC. Hybridisation was carried out in humidity chambers using the probe in a concentration of 100 ng/ml hybridisation buffer. After overnight incubation sections were washed in 2x SSC at 37°C followed by washes in 0.1x SSC/50% formamide 3 times for 7min. Hybridisation signals were detected using a Nucleic Acid Detection Kit (Boehringer Mannheim, Germany). Hybridised sections were processed as follows: sections were washed for 5min in buffer 1 (100mM Tris, 150mM NaCL, pH 7.5) at room temperature followed by incubation in buffer 1 containing 1% blocking mix for 30min. Anti-digoxigenin-antibody conjugate dilute 1:200 with buffer 1 containing 1% blocking mix was applied to each section at room temperature for 2h in a humidified chamber. Slides were then washed in buffer 2 (100mM Tris, 50mM MgCl, 100mM NaCl, pH 9.5) . A solution of 5-bromo-4-chloro-indolyl phosphate and nitrobluetetrazolium (obtained from Sigma, St Louis, MO, USA) in buffer 2 was made immediately prior to use, filtered and placed in a light-proof glass tray. Sections were developed overnight at room temperature. The sections were washed in TE-buffer (10mM Tris, 1mM EDTA, pH 8.0), counterstained with methylene green and mounted using gelatine/glycerol.

RNA isolation and Northern blot analysis

Total RNA from cultured cells or different tissues obtained from autopsy or biopsy were extracted with RNAzol B as suggested by the supplier. The following epithelial tissues were studied: tongue, pharynx, larynx, epiglottis, vocal fold, lung, duodenum, colon, liver, kidney, urethra, and inguinal skin. For Northern blot analysis 10 µg total RNA was fractionated on a denaturing 1% agarose gel containing formaldehyde following standard procedures [246] and blotted by capillary transfer on nylon membrane. After transfer, RNA was fixed to the membrane by ultraviolet irradiation (312 nm, 0.2 J/cm^2). Hybridisation was performed in phosphate buffer as previously described using a 0.42 kbp *Pvu*II/*Eco*RI fragment of the SKALP cDNA clone pGESKA as a probe [251]. Control hybridisations for equal loading were performed using a human 28S ribosomal RNA probe. All probes were labelled with ^{32}P by random priming following standard procedures. Autoradiography was done on X-Omat S film (Kodak, France) at -80°C with an intensifying screen.

Immunoelectron microscopy

Punch biopsies from normal and lesional psoriatic skin were fixed in 2% paraformaldehyde and divided into small pieces. Cryosubstitution and embedding in Lowicryl HM20 (Agar Scientific Ltd, Essex, England) were performed as follows: fixed tissues were stepwise infiltrated into 30% glycerol and frozen in liquid propane. Dehydration in methanol at -90°C for 48 h, and embedding in Lowicryl HM20 at -50°C were both performed in a cryosubstitytion apparatus (CS-auto, Riechert). Polymerization by indirect UV-light was performed for 48 h at -50°C, followed by 24 h at room temperature. Ultrathin skin sections on carbon coated copper grids were preincubated with 0.5% bovine serum albumin (BSA), 0.1% gelatine and 0.15% glycine in phosphate buffered saline (PBS). The grids were subsequently incubated with a polyclonal rabbit antiserum against recombinant

SKALP (1:500 in PBS/BSA) or the corresponding preimmune serum at the same dilution, at 4°C for 16 h. After washing the grids were incubated in protein A conjugated to 10 nm colloidal gold particles (1:100 in 1% BSA in PBS), for 60 min at room temperature. After washing the grids were counterstained with uranyl acetate (10 min) and lead citrate (1 min) according to standard protocols. The sections were examined in a JEOL jem1010 electron microscope at 80 kV.

Keratinocyte primary culture

Human epidermal keratinocytes were initially cultured according to the Rheinwald-Green system [224]. Primary cultures of keratinocytes were seeded on lethally irradiated (3000 rad, 3 min) Swiss mouse 3T3 fibroblasts in DMEM/F12 (3:1) (v/v) (Flow Laboratories, Irvine, Scotland) supplemented with 0.4 (g/ml hydrocortisone (Collaborative Research Inc. Lexington, MA, USA), isoproterenol (10^{-6} M) (Sigma, St. Louis, MO, USA), 100 U/ml penicillin plus 100 (g/ml streptomycin (Gibco, Breda, The Netherlands), 6% foetal calf serum (FCS) (Seralab, Nistelrode, The Netherlands), and 10 ng/ml epidermal growth factor (EGF) (Sigma, St. Louis, MO, USA). Cells were grown at 37°C, 95% relative humidity and 8% CO_2 in air. Liquid nitrogen stored keratinocytes from the primary culture were used in further experiments.

First passage and induction of keratinocyte differentiation

Keratinocytes were seeded at 10^5 cells in keratinocyte growth medium (KGM) as described before [229]. KGM was composed of keratinocyte basal medium (KBM, Clonetics, San Diego, CA, USA; 0.15 mM Calcium) supplemented with ethanolamine (0.1 mM) (Sigma, St. Louis, MO, USA), phosphoethanolamine (0.1 mM) (Sigma, St. Louis, MO, USA), bovine pituitary extract (BPE; 0.4% v/v) (Clonetics, San Diego, CA, USA), epidermal growth factor (EGF; 10 ng/ml) (Sigma, St. Louis, MO, USA), insulin (5 (g/ml) (Sigma, St. Louis, MO, USA), hydrocortisone (0.5 (g/ml) (Collaborative Research Inc. Lexington, MA, USA) , penicillin (100 U/ml) (Gibco, Breda, The Netherlands) and streptomycin (100 (g/ml) (Gibco, Breda, The Netherlands). For induction of differentiation, at confluence the culture medium was switched either to KGM supplemented with 5% FCS or to KGM depleted of growth factors (EGF, BPE, insulin) and hydrocortisone [246,256]. Non-differentiated keratinocytes, present as a confluent monolayer, were obtained by keeping the cells in KGM alone. After forty eight hours, the cultures were harvested either from 60 mm dishes (for RNA extraction) or from 24 well plates in which the cells were cultured on coverslips for immunocytochemistry.

Purification of PMN elastase; determination of anti-elastase activity

Human PMN elastase was purified from cells of a patient with chronic myeloid leukemia and calibrated as previously described [250]. Elastase activity and anti-elastase activity was measured using the fluorigenic substrate methoxy-succinyl-ala-ala-ala-pro-val-aminomethylcoumarin (Bachem, Bubendorf, Switzerland), as described previously [266]. The amount of inhibitor which reduces the activity of 1 nmol elastase with 50% under the given assay conditions, is defined as 1 unit of anti-elastase activity.

Cell detachment assays
Keratinocyte cultures on coverslips that were allowed to differentiate for 48 h, were extensively washed with PBS to remove all traces of the induction media. In order to investigate whether SKALP was secreted under these conditions, the culture medium was collected over a 6 h time period, and the amount of SKALP was determined by a sandwich-type enzyme-linked immunosorbent assay (ELISA) using a rabbit and a goat antiserum against recombinant SKALP. In addition the amount of anti-elastase activity in the medium was determined by a functional assay. PMN were isolated from peripheral blood according to standard procedures involving dextrane sedimentation, separation on a Ficoll-paque gradient and hypotonic lysis. This yielded a suspension of >95% pure PMN. Subsequently, the keratinocytes were exposed to PMN (2.10^6 per ml) activated with Ca-ionophore (1 μM), or to purified PMN elastase (10-25 μg/ml) in KGM supplemented with 1.8 mM Ca^{++}. PMN from two different donors were used. When keratinocytes were exposed to activated PMN, the effect of excess recombinant SKALP (50 μg/ml) or the addition of superoxide dismutase (500 U/ml) plus catalase 1000 U/ml) was tested. At regular intervals the coverslips were washed with PBS and fixed in ethanol for histological examination. The degree of cell detachment from the coverslip was quantitated by image analysis using the Vidas 2.1 system (Kontron, Germany), and expressed as a percentage of the total surface of the coverslip.

Results

SKALP is constitutively expressed in several squamous epithelia
We studied SKALP expression in 21 adult human tissues by immunohistochemistry (see figure 1). In these tissues the stroma was always negative, whereas the epithelium in a number of tissues studied showed positive staining. The following epithelial tissues were positive for SKALP expression: tongue, palate, lingual tonsil, gingiva, pharynx, epiglottis, vocal fold, esophagus, uterine cervix, vagina and hair follicle. The expression varied considerably from one weakly stained cell layer (as in vocal folds and hair follicles) to many intensely stained suprabasal layers (as in esophagus and vagina). Staining was never seen in the basal cells. When multiple layers were stained, the density of the cytoplasmatic staining increased with increasing differentiation, often in a distinct polarised (apical) localisation in the cytoplasm, while in other cases staining appeared to be localised close to the cell membrane.

Normal epidermis and skin adnexae are negative, except for the keratinocytes lining the epidermal part of the sweat gland ducts (fig 1a) and the infundibular part of the hair follicle (fig 1b). The suprabasal cells of psoriatic epidermis are strongly positive (fig 1c) as described previously [255]. The tongue dorsum was strongly positive: up to ten cell layers were intensively stained (fig 1d). In contrast the ventral part of the tongue was negative (not shown). The furrows surrounding the vallate papillae of the tongue stained intensily, but the taste buds were negative. In palate/lingual tonsil a distinct SKALP expression was observed in destroyed epithelial lining (fig 1e), while staining was absent

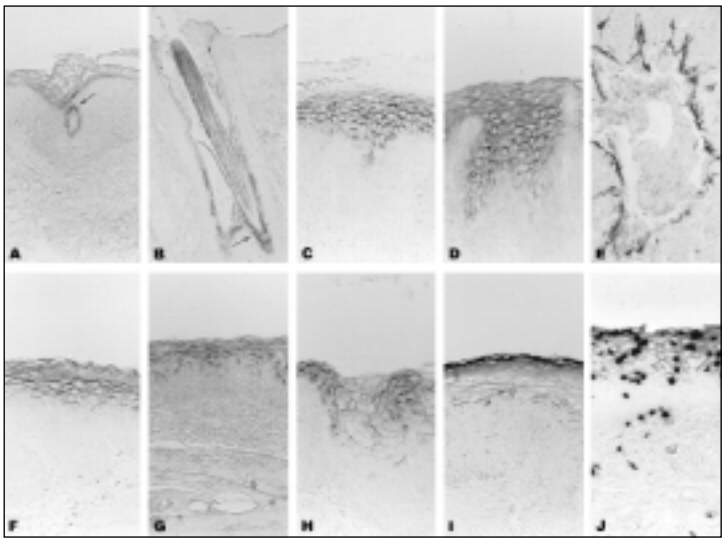


Figure 1. Immunohistochemical staining for SKALP in human epithelia. Staining was performed on formalin fixed paraffin sections, using a polyclonal rabbit antiserum. *(a)* normal epidermis is negative except for the keratinizing cells lining the sweat gland duct *(arrow)*; *(b)* hair follicle, positive cells are found in the infundibulum; in the following tissues SKALP is strongly present in the suprabasal cells: *(c)* psoriatic epidermis; *(d)* tongue; *(e)* tonsil; *(f)* gingiva; *(g)* epiglottis; *(h)* oesophagus; *(i)* vagina; *(j)* pharynx, doublestained for SKALP, which is present in the suprabasal epithelial layers, and for PMN elastase which is present as the intensely stained dots in the epidermis and the underlying dermis. Magnification 90x *(a,g)* and 180x *(b,c,d,e,f,h,i,j)*.

in the intact cover of the tonsil. Keratinising gingiva was moderately positive for three or four most differentiated cell layers (fig 1f). The lining of the epiglottis was positively stained in multiple layers (fig 1g). In between, areas of pseudostratified columnar cells, with ciliated cells common to respiratory epithelia, were negative with respect to expression of SKALP. True vocal fold was slightly positive (not shown). Oesophageal lining, showed many positive cell layers, especially the superficial layers which were intensively stained in a polarised pattern (fig 1h). The uterine cervix and vagina were positive for multiple cell layers, with varying intensity. The staining appeared to be more cell membrane associated than cytoplasmatic (fig 1i). In most of these epithelia, infiltrating mononuclear cells and few PMNs are found in the connective tissue or in the epithelium itself, and their presence appears to be physiological. In pharynx we found a considerable number of PMN present. Fig 1j shows a double staining for SKALP and PMN elastase. In addition to the tissues shown in figure 1a-j we examined a number of epithelial and non-epithelial tissues that were negative for SKALP expression, such as brain, cornea, larynx, lung, bronchus, duodenum, colon, liver, kidney, urethra, and skin from various body regions (not shown).

Because SKALP contains a number of repeats in the N-terminal domain that can be used as TGase substrate a limited number of tissues was examined for type 1 TGase. The known

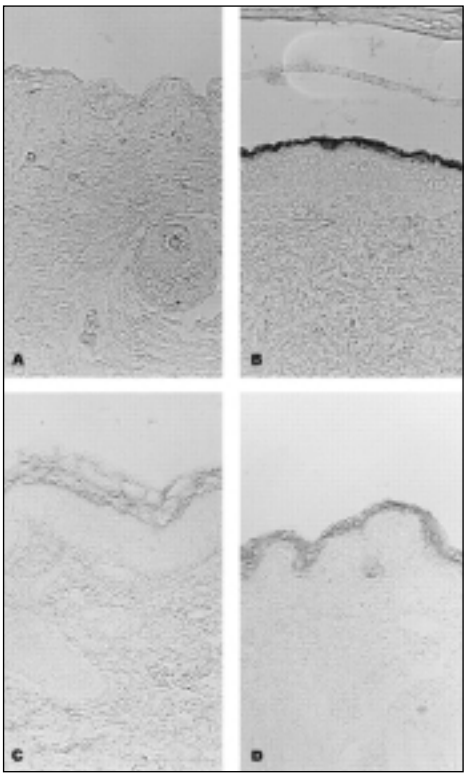
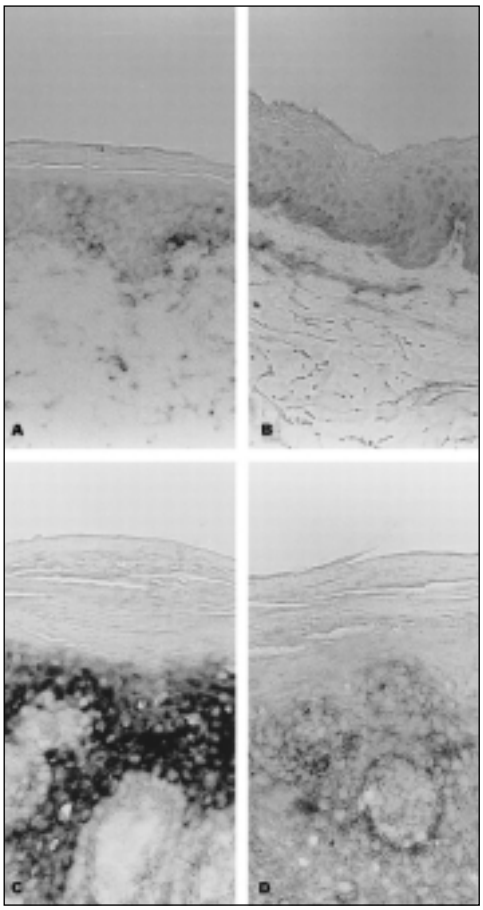


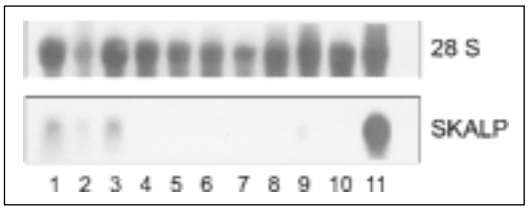
Figure 2. SKALP expression in foetal epithelia. Staining was performed on formalin fixed sections. Foetal epidermis was negative up to week 20 (fig 2a). From week 27-28 onwards strong epidermal staining was seen in the suprabasal cells (figure 2b). SKALP expression was rapidly downregulated after birth. In neonatal epidermis after 1 month a faint staining was visible in the upper layer of the stratum granulosum (figure 2c) and SKALP was undetectable at 3 months (as in adult skin). In oral epithelia SKALP was expressed as early as week 17 (fig 2d) and continued to be expressed during adult life (not shown). Magnification 90x

tissue distribution [267] and our own observations indicate that in psoriatic epidermis, oesophagus and oral epithelia SKALP and TGase co-localise in the upper half of the suprabasal compartment (not shown).

SKALP is developmentally regulated in foetal epidermis

Foetal tissues (9, 17, 22 and 28/29 wk gestation) and neonatal tissues (1 and 3 months *post partum*) were stained for SKALP. We studied the expression in oral epithelia, that were shown to be positive in the adult, and in epidermis, which was negative in the adult (see above). For comparison we stained the tissues for other known differentiation-related markers such as CK 10, CK 13/16 and involucrin (data not shown). In a 9 wk old embryo, which could be examined *in toto*, no SKALP could be detected in any tissue. The periderm which is at this timepoint starting to show the first signs of stratification was also negative for CK 10, CK 13/16 and involucrin. At 22 weeks gestation, CK 10, CK 13/16 and involucrin positive cells are found in the upper suprabasal layer of the epidermis, whereas SKALP is absent at this timepoint (fig 2a). At 28 weeks, SKALP expression is found in the granular layer and upper spinous layer of the interfollicular epidermis (fig 2b). At this timepoint the morphology of the epidermis is similar to adult human epidermis, including full stratification, keratinisation, suprabasal expression of CK 10 and involucrin, and





↑ Figure 3. Northern blot of SKALP mRNA in various adult tissues. Lower panel. A 0.8 kb message was found in tongue (lane *1*), epiglottis (lane *2*), pharynx (lane *3*), vocal fold (lane *9*) and in lesional psoriatic skin which was used as the positive control (lane *13*). Larynx, lung, kidney, urethra, skin of the inguinal region, and liver (lanes *4, 5, 6, 7, 8* and *10* respectively) were negative. In the upper panel a probe for 28S ribosomal RNA was used for control hybridizations to check for equal RNA loading.

← Figure 4. In situ hybridization of SKALP mRNA in human epidermis. Cryosections of normal human skin and lesional psoriatic skin were used for in situ hybridization with a digoxigenin-labelled SKALP cRNA probe. Normal skin was negative both with the antisense and sense probe *(figures 4a and 4b)*. Lesional psoriatic skin was strongly positive in the suprabasal cells, using the antisense probe *(figure 4c)*. No signal above background was detected with the sense probe *(figure 4d)*. The occasional alkaline phosphatase precipitate adjacent to the basal membrane is non-specific, and was seen both with the sense and antisense probe. Magnification 90x

the absence of CK 13/16 in the suprabasal cells, as previously described by others [268,269]. In neonatal epidermis one month *post partum* the expression of SKALP is still faintly detectable in the upper layer of the stratum granulosum and in the stratum corneum (fig 2c). In neonatal epidermis 3 months *post partum* SKALP expression is completely down-regulated as in normal adult skin. The expression pattern of CK 10 remains unchanged during the postnatal period. Involucrin expression, which was found in the foetal stratum granulosum and stratum spinosum, is only found in the stratum granulosum of adult epidermis. In adult interfollicular epidermis SKALP is completely absent, but is re-expressed during the hyperproliferative differentiation program as seen in psoriasis (see fig 1c) and injury, where it is always co-expressed with CK 16. Remarkably, in the foetal and neonatal epidermis that was positive for SKALP no positive staining was seen with the Ks8.12 (anti-CK 13/16) antibody, indicating that SKALP and CK 16 -at least in foetal development- are subjected to distinct regulatory mechanisms.

In addition to epidermis we investigated the expression of SKALP, CK 10, 13 /16 and

involucrin in foetal and neonatal tongue and tonsils. SKALP was found in the suprabasal keratinocytes of tongue epithelium as early as 17 weeks gestation (fig 2d). In the lingual tonsils SKALP expression was found from 28 weeks onwards (no material was available from earlier time points). In contrast to epidermis, SKALP expression in these epithelia was not downregulated in neonatal skin, but continued to be present in adult life, as shown in fig 1.

Expression of SKALP at the mRNA level

Fresh autopsy material was obtained, stored at -80 °C, and used for northern blot analysis. In tongue, pharynx and psoriatic skin a strong expression of SKALP could be demonstrated whereas in epiglottis and vocal fold moderate levels were present (fig 3). In larynx, lung, kidney, urethra and normal skin no SKALP mRNA was found. These findings correlated with the expression of SKALP at the protein level, as found with immunohistochemistry.

Because our studies suggested a discrepancy with data from Nonamura *et al* who reported the focal expression of SKALP in normal adult epidermis as shown with *in situ* hybridisation, we have examined epidermis from many different locations. We were unable to demonstrate SKALP expression at the protein level in adult epidermis from ear, breast, axilla, scrotum, mamilla, palms and soles (not shown). Using mRNA *in situ* hybridisation on frozen sections of normal and psoriatic skin from the trunk, our results paralleled the findings with immunohistochemistry (fig 4). With the antisense cRNA probe a strong expression was found in the suprabasal compartment of psoriatic epidermis, whereas normal epidermis was negative. All tissues were negative with the sense cRNA probe. Our findings at the mRNA level are in agreement with the findings at the protein level as shown in fig 1.

SKALP is present in secretory vesicles of epidermal keratinocytes

Electron microscopic examination of normal and lesional psoriatic epidermis revealed a positive staining of suprabasal keratinocytes and the stratum corneum of psoriatic epidermis. No significant signal was present in keratinocytes from normal epidermis or in melanocytes, which is in accordance with the findings at the light microscopical level. Staining with the preimmune serum was negative (figure 5a). As shown in figure 5d staining was mainly observed in the intercellular spaces of the stratum corneum and over the lamellar granules of the keratinocytes in the upper spinous and granular layer (fig 5b). In addition, intracellular labelling could also be observed over small vesicular structures and in the Golgi apparatus of these cells (not shown). Since these structures are implicated in secretion, these findings indicate synthesis and secretion of SKALP from suprabasal keratinocytes.

Expression of SKALP protects against PMN-elastase mediated cell detachment

The specificity of SKALP for the PMN-derived proteinases elastase and proteinase 3, and its constitutive expression in epithelia that are subjected to inflammatory stimuli suggests a role in the control of inflammation. We therefore investigated whether SKALP expres-

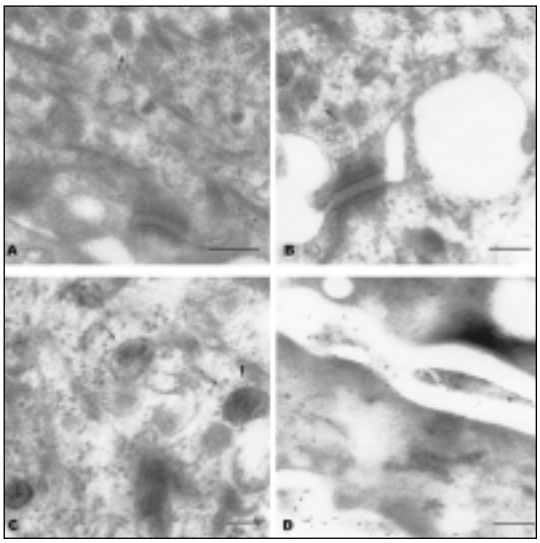


Figure 5. Immunoelectron microscopy of SKALP in human epidermis. *(a)* psoriatic epidermis stained with pre-immune serum. An area of a keratinocyte of the spinous layer is shown, near a desmosome. No immunogold labelling was seen in any of the structures including the lamellar granules *(arrow)*. *(b)* psoriatic epidermis stained with anti-SKALP serum. A group of labelled lamellar granules is shown *(arrow)* near a desmosome, at the cell membrane. *(c)* high power magnification of the same preparation as in *(b)*, with a labelled lamellar granule *(arrow)* and an empty lamellar granule (arrowhead). *(d)* stratum corneum of psoriatic epidermis stained with anti-SKALP serum. Clear labelling of the outer layers of the corneocytes is visible. Bar=100nm

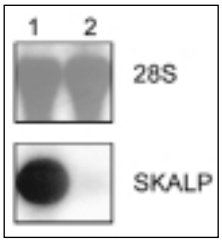


Figure 6. Northern blot analysis of SKALP expression in cultured keratinocytes. Human epidermal keratinocytes were induced to differentiate either in KGM depleted of growth factors (KGM/-GF) or in KGM with fetal calf serum (KGM/FCS). In KGM/-GF (lane *2*) SKALP is not expressed, whereas a strong signal is seen in KGM/FCS (lane *1*). In the upper panel control hybridization with 28S ribosomal RNA is shown to check for equal loading.

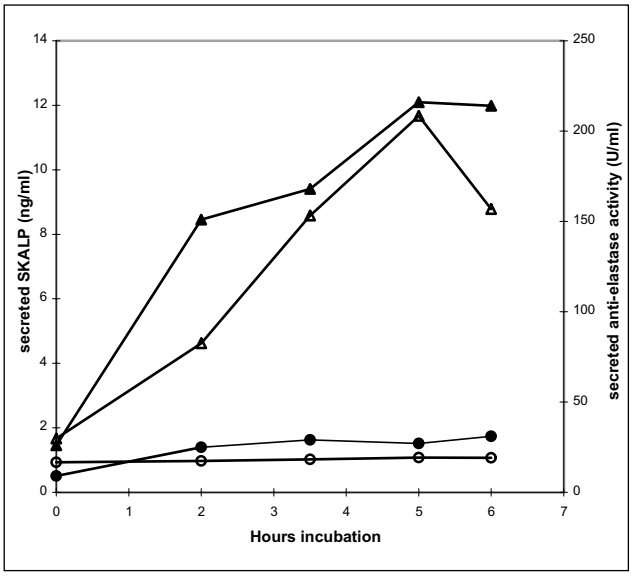


Figure 7. Secretion of SKALP in the medium of cultured keratinocytes. After induction of diferentiation in KGM/FCS or in KGM/-GF, the cells were extensively washed, and incubated in KGM supplemented with 1.8 mM Ca++, which is the medium used for detachment assays (see below). During a 6 h time course, secretion of SKALP in the medium was measured at the protein level by ELISA (left-hand Y-axis, open circles and triangles) or measured functionally and expresssed as units anti-elastase activity per ml (right-hand Y-axis, closed circles and triangles). Under these conditions the keratinocytes cultured in KGM/FCS (triangles) continued to secrete SKALP whereas the keratinocytes cultured in KGM/-GF (circles) remained negative.

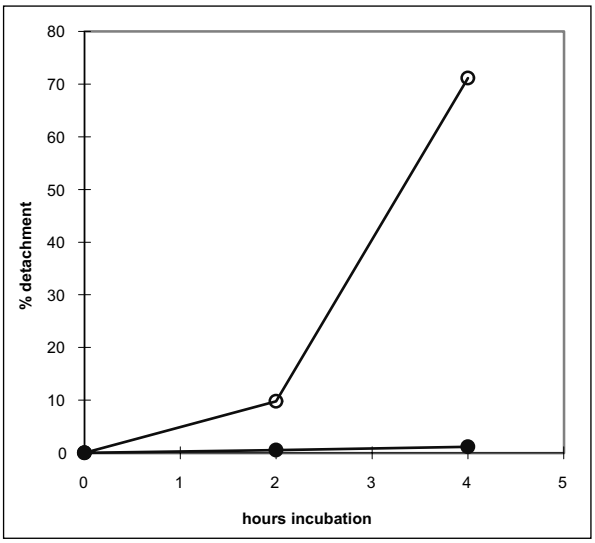


Figure 8. Time course of cell detachment by purified PMN elastase. Human epidermal keratinocytes cultured on coverslips in KGM/-GF (open circles) or in KGM/FCS (closed circles), were extensively washed, and subsequently exposed to PMN elastase (10 µg/ml) in KGM with 1.8 mM Ca++. After 2 and 4 h cells were washed, fixed and stained with H&E. Cell detachment was quantitated by image analysis.

sion could protect against elastase-dependent cell damage in an *in vitro* system. Human epidermal keratinocytes were cultured in KGM to form a confluent monolayer. At this stage, no differentiation is induced and SKALP is not expressed. Differentiation was induced either by growth factor depletion [265] or by the addition of foetal calf serum [246]. KGM depleted of growth factors (KGM/-GF) induces a differentiated phenotype, as assessed by type 1 TGase expression, and resembles normal skin as witnessed by the expression of CK 1/10 and the absence of SKALP [77]. KGM with foetal calf serum (KGM/FCS) induces differentiation, as assessed by the expression of type 1 TGase, with a psoriasiform phenotype such as sustained hyperproliferation and expression of SKALP [77]. Figure 6 shows the expression of SKALP in these culture systems at the mRNA level. After induction of differentiation, these cultures were washed extensively to remove the induction media and were placed in the culture medium that was used in subsequent experiments for co-culture with PMN (KGM with 1.8 mM Ca^{++}, without serum). The secretion of SKALP in the medium was measured by ELISA and in a functional assay as anti-elastase activity. Figure 7 shows that during a 6 h time-course SKALP is secreted in the medium by cells that were allowed to differentiate in KGM/FCS, whereas the cells differentiated in KGM/-GF were negative. Having established that the culture models differed in SKALP expression, the keratinocytes were exposed to purified human PMN elastase. During a 4 h time course, cell detachment from the coverslips was visualised by H&E staining and quantitated by image analysis (figure 8 and 9). Keratinocytes cultured in KGM/FCS (positive for SKALP synthesis and secretion) were found to be protected against elastase-dependent detachment, whereas cells cultured in KGM/-GF (negative for SKALP synthesis and secretion) were fully detached from the tissue culture dish. Elastase-dependent loss of adhesion was concentration dependent (not shown). Next we investigated whether activated PMN could also induce the effects seen with the purified enzyme. Differentiated

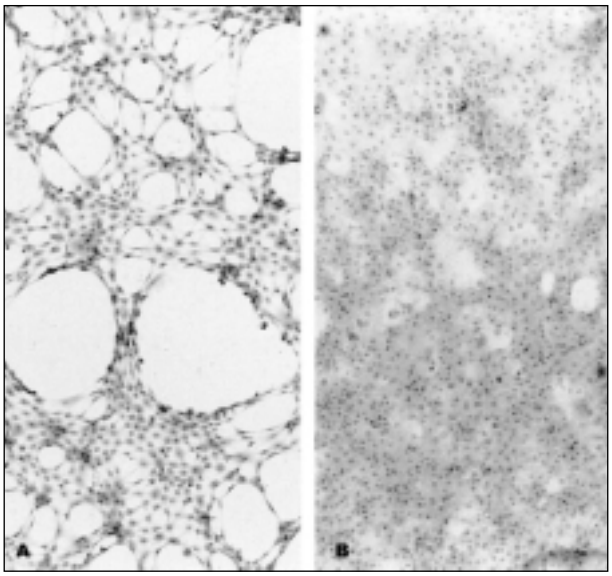


Figure 9. H&E staining of keratinocyte cultures exposed to PMN elastase. Human epidermal keratinocytes cultured on coverslips in KGM/-GF or in KGM/FCS, were extensively washed, and subsequently exposed to PMN elastase (10 μg/ml) in KGM with 1.8 mM Ca^{++}. Cells were washed, fixed and stained with H&E. After 4 hours, cell detachment and retraction was seen in keratinocytes cultured in KGM/-GF *(fig 9a)* whereas no signs of cell detachment were visible in cells cultured in KGM/FCS *(fig 9b)*. Magnification 45x

cultures of keratinocytes were extensively washed and exposed to calcium-ionophore activated PMN. Experiments were performed in duplicate with PMN from two different donors. Within 12 hours, activated PMN caused cell detachment in keratinocyte cultures grown in KGM/-GF, although to a lesser extent than observed in the concentration range of purified elastase (approximately 10-15 % detachment after 12 hours). The SKALP-expressing keratinocytes (grown in KGM/FCS) were fully protected against cell detachment (figures 10 and 11). Addition of recombinant SKALP (50 μg/ml) to keratinocytes cultured in KGM/-GF gave complete protection against PMN-mediated damage (figure 11e), whereas addition of SOD (500 U/ml) plus catalase (1000 U/ml) was only marginally effective (figure 11d).

Discussion

The proteinase inhibitor SKALP/elafin was first discovered at the biochemical level in psoriatic skin, which is reflected in the acronym SKALP [250], as a specific inhibitor of leukocyte elastase which is reflected in the name elafin [239]. Retrospectively, both these names are unfortunate, since we now know, firstly, that SKALP is not normally present in epidermis but is constitutively expressed in other epithelia, and secondly that the inhibitory

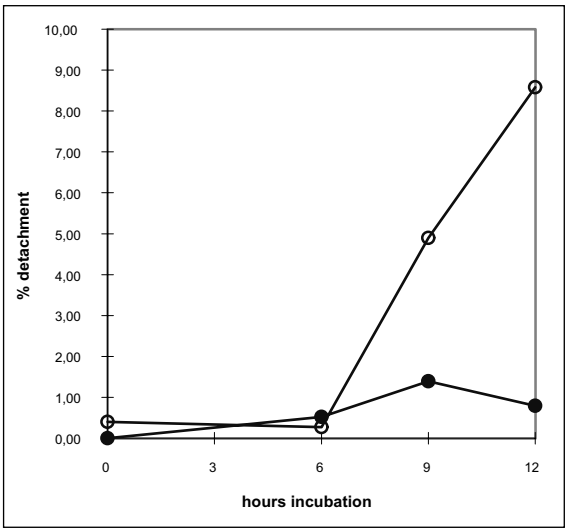


Figure 10. Time course of cell detachment by activated PMN. Human epidermal keratinocytes cultured on coverslips in KGM/-GF or in KGM/FCS, were extensively washed, and subsequently exposed to PMN (2.10^6/ml) activated by Ca-ionophore (1 μM).). Experiments were performed in duplicate with PMN from two different donors. The coverslips were washed, fixed and stained with H&E. Cell detachment was quantitated by image analysis. Between 6 and 12 h incubation, cells detached from the cultures that did not express SKALP (keratinocytes cultured in KGM/-GF, open circles), whereas the SKALP expressing culture (keratinocytes cultured in KGM/FCS, closed circles) was fully protected.

profile of SKALP also includes leukocyte proteinase-3 [254]. In view of its specificity for leukocytic proteinases, it was hypothesised that SKALP is involved in the regulation of cutaneous inflammation either by interfering with PMN migration or by providing protection against excessive proteolysis of extracellular matrix components. In previous studies we have documented the expression of SKALP in psoriatic epidermis, in injured skin and in cell culture [246]. A striking parallel was found with the expression pattern of CK16, which is not normally expressed in the interfollicular epidermis but is induced in psoriasis, after injury and in cell culture [6,270]. We hypothesised that both SKALP and CK16 were part of the hyperproliferative differentiation program of normal epidermis, and subjected to similar control mechanisms. In the present study we show that SKALP and CK16 are not co-expressed during foetal and neonatal development. CK16 is not expressed in any stage of development of foetal epidermis as previously described by others [269], whereas SKALP is expressed in the third trimester and downregulated in neonatal skin. In contrast to previous reports that dealt with SKALP in epidermal keratinocytes, either in pathology or in cell culture, here we demonstrate SKALP to be expressed in 'normal' squamous epithelia. The adjective 'normal' in this respect should be used with caution. Several normal tissues are subjected to continuous mechanical stress or inflammatory stimuli, which may influence the structure and organisation of the lining epithelium. In normal epiglottis, squamous metaplasia can be observed, that is partly functional or due to mechanical stress. Pathogens may be present in the pharynx of healthy individuals, and in normal pharynx and esophagus PMN infiltration is not considered to be pathological. Also in the infundibular part of the hair follicle the presence of bacteria that may induce an inflammatory response is common. SKALP expression was demonstrated in the tonsillar crypts where destruction of the epithelial lining by lymphocytes was observed. Lymphocytes transmigrate the tonsillar epithelium into the crypts that also

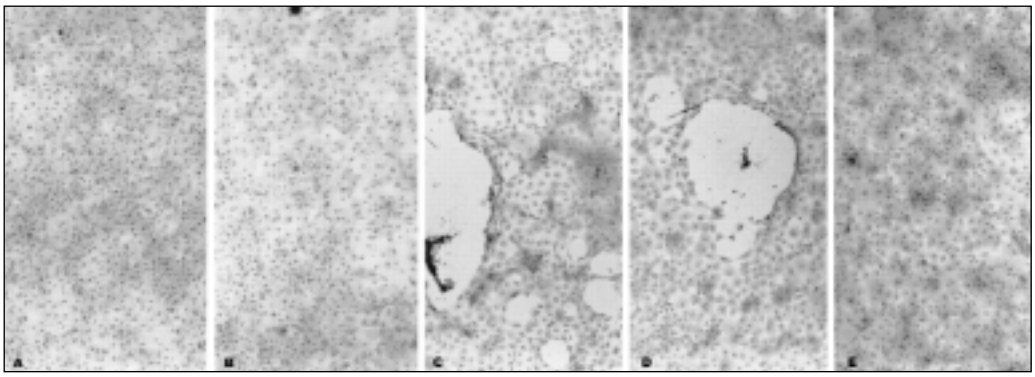


Figure 11. H&E staining of keratinocyte cultures exposed to activated PMN. Human epidermal keratinocytes cultured on coverslips in KGM/-GF or in KGM/FCS, were extensively washed, and subsequently exposed to PMN (2.10^6/ml) activated by Ca-ionophore (1 μM). After 12 h incubation, the cells were washed, fixed and stained with H&E. Figure 11a shows a confluent culture (KGM/FCS) after 12 h without PMN, which was taken as the control value. No detachment was seen in these cultures; this was also found for the KGM/-GF cultures (not shown). SKALP-expressing cultures (KGM/FCS) exposed to activated PMNs did not show significant detachment of cells (figure 11b), whereas in the cultures that lacked SKALP-expression (KGM/-GF) considerable cell detachment was noted (figure 11c). Recombinant SKALP (50 (g/ml) could completely prevent cell detachment in the KGM/-GF cultures (figure 11e) whereas addition of SOD (500 U/ml) plus catalase (1000 U/ml) had only a marginal protective effect (figure 11d).

contain the desquamated cells of the stratified squamous epithelium. This passing through is accompanied by desolution of the epithelium, which may be the trigger for SKALP induction as part of the ongoing inflammatory response.

SKALP is specific for elastase and proteinase 3 that are proteinases present in PMN. In addition, elastase is found in monocytes, albeit at lower levels than in PMN. Continuous trafficking of PMNs and mononuclear cells to epithelia could expose these tissues to breakdown of extracellular matrix proteins as is known from various pathological conditions [266,271]. Studies by several authors have shown that PMN can *in vitro* degrade extracellular matrices and cause cell detachment by distinct mechanisms involving proteolytic enzymes or oxidative molecules [272-275]. Recently it was shown that activated PMN can express cell-surface bound elastase and cathepsin G which provides a potent mechanism to egress from the vasculature, penetrate tissues and migrate to sites of inflammation [273]. Using cultured keratinocytes exposed to purified PMN elastase or to activated PMN we show that expression of SKALP provides protection against elastase-mediated detachment *in vitro.* In this model system we used Ca-ionophore activation of PMN which is a powerful stimulator of lysosomal enzyme secretion (up to 30% of the total elastase content) and gives a moderate superoxide production. After 9 h of exposure to activated PMN a cytopathic effect was seen on keratinocytes that lacked SKALP expression. The cell damage during this period was clearly elastase/proteinase 3 dependent since the addition of recombinant SKALP provided protection. In contrast, a high concen-

tration of SOD and catalase did not protect against cell detachment and retraction. SKALP could protect against elastase mediated damage in two ways. The presence of a signal peptide and its presence in secretory vesicles and lamellar bodies suggests that SKALP is secreted in the extracellular space, where it can neutralise its target proteinases. On the other hand, the TGase substrate motifs of SKALP enable crosslinking to the pericellular matrix and the cornified envelope. SKALP/elafin was recently found to be crosslinked to loricrin, a component of the cornified envelope [258], suggesting that SKALP is crosslinked to intracellular proteins, and can also act as an anchored proteinase inhibitor. We have recently obtained indirect evidence that SKALP is involved in protection of epidermal integrity in pustular forms of psoriasis [261]. It was shown that in forms of pustular psoriasis, which are characterized by a massive influx of PMN, the amount of epidermal SKALP is much lower than in plaque type psoriasis. It remains to be established whether this phenomenon is caused by an intrinsic (genetic) defect in these patients, or by inactivation/clearance of SKALP.

Assuming that induction of SKALP controls excessive proteolysis in adult skin, the function of foetal SKALP expression is not clear at the moment, since no obvious signs of inflammation are present in the foetal skin itself. It was, however, recently reported that at the end of gestation increased levels of inflammatory cytokines are found in the amion fluid, and evidence for an inflammatory response has been found in the forebag compartment of the uterus presumably as a result of leakage of microbial stimuli from the vagina and cervix into the uterus [276]. Speculatively, SKALP expression could be induced via this route to ensure protection of the foetal skin against proteolytic activity in the amnion fluid at the end of gestation. However, no evidence for the presence of inflammatory cells or proteinases derived thereof in the amnion fluid, has been published to our knowledge. Alternatively, SKALP induction could be a mechanism to provide temporary protection to the epidermis of the new-born, when switching from the foetal submerged, sterile environment to a dry environment which allows colonisation of the skin with micro-organisms. Although a fully keratinised epidermis is present at term, the barrier function of neonatal skin is incomplete [268]. The observed downregulation of SKALP in neonatal epidermis could therefore be related to the functional maturation of barrier function. It has to be noted that under conditions that compromise barrier function such as tape stripping and psoriasis, SKALP is again induced. Recently Nonamura *et al* have described the focal presence of SKALP mRNA in normal epidermis, using *in situ* hybridisation [277]. Here, we have studied a large number of samples from various locations of normal skin, but we failed to demonstrate significant expression of SKALP at the protein level. Also with *in situ* hybridisation we were unable to demonstrate the presence of SKALP in normal skin. We do occasionally observe SKALP expression in the epidermis near hair follicles and acrosyringia, which may explain the focal expression found by Nonomura *et al* [277].

Remarkably, SKALP in tongue and tonsil epithelium is induced much earlier than in epidermis and continues to be expressed in the neonatal phase and throughout adult life. In many of the moist oral epithelia (and of course in the tonsils) the presence of inflammatory cells is physiological, as a result of continuous exposure to environmental and microbial stimuli. In adult epidermis which is a dry, keratinising epithelium, inflamma-

tory cells are not normally present, which may explain the difference in SKALP expression.

On the basis of the pattern of SKALP expression, one would surmise that SKALP expression is induced by inflammatory cells such as PMN. Interestingly, Sallenave et al. demonstrated induction of SKALP expression in tumour cell lines by the cytokines interleukin 1 (IL-1) and tumour necrosis factor alpha (TNF-α), but also by the PMN-derived proteinases human leukocyte elastase and cathepsin G [278]. This finding is in line with that of Perlmutter et al. who reported that neutrophil elastase regulates the synthesis of its inhibitor (1-proteinase inhibitor in human monocytes and bronchoalveolar macrophages [279]. The cytokines IL-1 and TNF-α are considered to be initiating cytokines in inflammatory processes in cutaneous tissue [271]. In normal tissues where continuous monitoring of the tissue by inflammatory cells is desired for reasons of host defence, this control mechanism might explain SKALP presence in epithelia such as esophagus, pharynx, tongue and vagina. The difference in SKALP expression in the various epithelia could be related to differences in inflammatory stimuli to which the tissues are subjected. However, ultimate proof for its supposed relevance in control of epithelial inflammation has to come from studies using transgenic mice lacking a functional SKALP gene, which will be the direction for future research.

SKALP/Elafin gene polymorphisms are not associated with pustular forms of psoriasis

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Abstract

Psoriasis is a multifactorial skin disease characterised by epidermal abnormalities and infiltration by lymphocytes and polymorphonuclear leukocytes (PMN). SKALP, also known as elafin, is a potent inhibitor of human leukocyte elastase and proteinase 3, two PMN-derived proteinases implicated in tissue destruction and leukocyte migration. We have shown that, at least at the protein level, SKALP is significantly decreased in lesional skin of patients with pustular psoriasis compared to plaque type psoriasis. This finding raised the possibility that SKALP could be one of the candidate genes for pustular forms of psoriasis. We therefore performed SSCP analysis on the SKALP gene to screen for mutations/polymorphisms in the exons of 30 patients with plaque type psoriasis, 15 patients with pustular psoriasis and 48 healthy controls. In exon 1 a polymorphism was detected at position +43 relative to the translation start site, resulting in a substitution of threonine for alanine in the signal peptide. In the promoter region a dinucleotide repeat polymorphism was identified. Both polymorphisms were not associated with pustular psoriasis, or psoriasis in general. Our data indicate that the decrease in SKALP activity in pustular psoriasis is not caused by mutations in the coding region of the gene, and that there is no allelic association between pustular psoriasis and SKALP gene polymorphisms.

Introduction

Psoriasis is a polygenic, chronic inflammatory skin disease of unknown aetiology. An underlying autoimmune process or a defect in keratinocyte growth control mechanisms has been implicated in the pathogenesis of this disease [280,281]. A genetic base for psoriasis is likely as indicated by family and twin studies [282]. In a study of 61 monozygotic twins a concordance of 73% has been reported, indicating a major genetic component in psoriasis [283]. Recently, several groups have started genome-wide searches for genes conferring susceptibility to psoriasis [284-287]. Loci on chromosomes 2 [286], 4 [287], 8 [286], 17 [284], 20 [286] and within the MHC cluster on chromosome 6p21 were found to be linked with psoriasis of the chronic plaque type. Psoriasis pustulosa palmoplantaris is associated with and increased frequency of HLA B8 [288] and HLA DR9 [289]. So far no mutations or polymorphisms in individual genes have been found that are linked to plaque psoriasis or pustular psoriasis.

Histologically, psoriasis is characterised by a disturbed epidermal proliferation and differentiation. In the psoriatic lesion a dense dermal infiltrate is present, composed of lymphocytes and polymorphonuclear leukocytes (PMN). PMN can also invade in the epidermis, to form microabcesses of Munro or spongiform micropustules of Kogoj. A subform of psoriasis is pustular psoriasis, which can present itself as a localised form (psoriasis pustulosis palmoplantaris, acrodermatitis continua of Hallopeau) or a generalised form (generalised pustular psoriasis of von Zumbusch). The main characteristic of these conditions is the presence of macroscopic pustules, which are the result of an extreme epidermal infiltration of PMN. The pathogenetic events leading to the formation of

macropustules in these diseases are unknown. Both an increase of epidermal chemotactic factors [290,291] and an increased chemotactic response of the PMN [291] have been implicated as a factor contributing to the massive PMN influx.

PMN-derived neutral proteinases such as elastase, proteinase 3 and cathepsin G are important inflammatory mediators that have been implicated in tissue damage and PMN migration. The action of elastase and proteinase 3 is controlled by systemic proteinase inhibitors like alpha-1-proteinase inhibitor and by locally produced proteinase inhibitors like SKALP [238], also known as elafin [293]. SKALP is a member of the Trappin gene family that we have recently described [241]. In normal epidermis SKALP is absent but it is induced under inflammatory conditions [237,238]. We have recently shown *in vitro* that SKALP is a major inhibitor involved in the protection of the epidermis against PMN-dependent proteolysis [294]. Because of the pronounced presence of PMN in the epidermis of patients with pustular forms of psoriasis, we hypothesised that deregulation of epidermal anti-protease activity could contribute to PMN chemotaxis and tissue destruction. We have recently shown that, at least at the protein level, SKALP is significantly decreased in lesional skin of pustular psoriasis compared to plaque type psoriasis [261]. These findings raised the possibility that SKALP could be one of the candidate genes for pustular forms of psoriasis. We therefore performed single strand conformation polymorphism (SSCP) analysis to screen for mutations/polymorphisms in the exons 1 and 2 of the SKALP gene of 30 patients with plaque type psoriasis, 15 patients with pustular psoriasis and 48 healthy controls. Here we report a polymorphism in exon 1, which results in an amino acid substitution. However, this polymorphism was equally distributed in the patient and in the control population. Similarly, we found a polymorphic CA-repeat in the promoter region which was not associated with pustular psoriasis, or psoriasis in general.

Materials and methods

Patients

Five ml blood samples in ethylene diamine tetra-acetic acid (EDTA) coated containers were collected from 30 patients with chronic plaque psoriasis and 15 patients with a pustular form of psoriasis, either localised or generalised (pustulosis palmoplantaris n=12, acrodermatitis continua of Hallopeau n=1, generalised pustular psoriasis von Zumbusch type n=1, non-generalised pustular psoriasis n=1). All patients had active disease. Furthermore, blood samples were obtained from a three-generation family and from 48 healthy controls, the latter via the transfusion department. Prior to analysis, blood samples were stored at -80˚C.

Polymerase chain reaction amplification

Genomic DNA was isolated from peripheral blood lymphocytes by standard methods [295] and used as a template for amplification of exons 1 and 2 of the SKALP gene, coding for the primary translation product. When the 5'non-coding region of the SKALP gene was sequenced a dinucleotide repeat in the promoter region of the gene was noted, which was

polymorphic. For amplification of the regions of interest, oligonucleotide primers (Table 1) were synthesised on the basis of intronic sequences and the nuclotide sequence of the flanking regions of the dinucleotide repeat (Isogen Bioscience, Maarssen, the Netherlands). Amplifications were performed in a total volume of 25 (l containing 200 ng genomic DNA, 5-10 mM $MgCl_2$, 50 mM KCl 10 mM Tris, pH 8.3, 0.2 mM of each dNTP, 10 pM of each primer and 0.5 U Taq DNA polymerase. The PCR cycling profile for all PCR reactions was as follows: preheating to 95˚C for 5 min, followed by 35 cycles of 94˚C for 30 sec, annealing temperature for 1 min (Table 1), 72˚C for 2 min. The PCR was ended with a final extension at 72°C for 10 min. All PCR reactions were carried out in a Peltier-Thermal cycler (MJ Research, Massachusetts, USA).

Table 1

Primers[a]	Sequence[b]	Annealing temperature (˚C)	Size (bp)
EXON 1 For1 (pos. -46 to -25) Rev1 (pos. +103 to +84)	 AAGATTGGTATGGCCTTAGCTC CCAGCAGGTCACCTGTTCAC	60	150
EXON 2 For2 (pos. +897 to +917) Rev2 (pos. +1181 to +1163)	 GCTTACTGGGTATAAATGTGG AGCCTTCACAGCACTTCTT	53	285
For3 (pos. +1049 to +1068) Rev3 (pos. +1236 to +1217)	CCAGTCAAAGGTCCAGTCTC TCGTTCTCAAGCTAGTGCTC	57	188
REPEAT PROMOTER For4 (pos. -989 to -968) Rev4 (pos. -834 to -851)	 ACCTTTTCCAGAAGAAGGAACC CCTGACCCCTGACCACAG	58	156

a Numbers in brackets are the 5' base position from the translation start site.
b Primer sequences are written 5' to 3'.

Non-radioactive single strand conformation polymorphism (SSCP) analysis
Conformation polymorphisms were visualised by SSCP analysis [296,297]. For denaturation of the DNA, 3.5 (l of the PCR product was mixed with 7 µl of 50 mM NaOH and 1 mM EDTA, heated at 94˚C for 5 min and subsequently placed on ice. Five µl of this mixture was loaded in 8 µl wide slots on a CleanGel polyacrylamide gel (Cleangel 10%, Pharmacia Biotech, Uppsala, Sweden), which was rehydrated in 112 mM Tris-acetate, pH 6.4 with 0.006% bromophenol blue. Electrophoresis was carried out for each PCR product at two different temperatures (4˚C and 20˚C) at 10 W for 20 min, and 20 W for 60 min in 0.2 M Tris 0.2 M Tricine 0.55% (w/V) SDS, pH 8.3. Silver staining was performed according to the manufacturer's guidelines: After fixation of the gel for 30 min in 10% acetic acid solu-

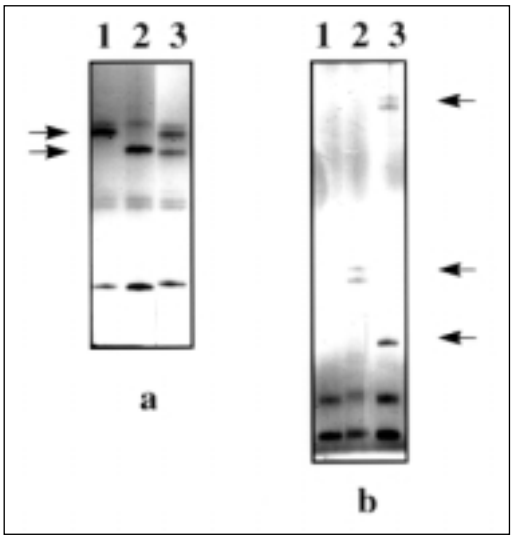

Figure 1. Non-radioactive SSCP analysis of exon 1 *(a)* and the dinucleotide repeat in the 5' upstream region *(b)* of the SKALP gene. *(a)* Exon 1. Different migration patterns were seen. Further analysis revealed an A → G polymorphism at position +43 from the transcription start site. Lane 1, homozygous A, lane 2 homozygous G, lane 3 the heterozygous pattern. *(b)* Dinucleotide repeat in 5' upstream region. Different migration patterns were seen. Further analysis revealed two additional CA repeats. Lane 1 homozygous for the allele with 6 CA-repeats, lane 2 the heterozygous pattern showing 2 extra bands, lane 3 the single patient showing an aberrant pattern. Arrows mark the different allelic bands.

tion, the gel was washed 3 times in demineralised water. Subsequently, staining with 0.1% $AgNO_3$ with 0.037% formaldehyde was performed during 30 min followed by washing with demineralised water and development of the gel using 2,5% Na_2CO_3 with 0.037% formaldehyde and 0.002% Na-thiosulphate. Development was stopped with 2% glycine 0.5% EDTA-disodium solution and gels were impregnated with 5% glycerol.

Cloning and DNA sequence analysis

In exon 1 and in the promoter region variations in the mobility patterns in SSCP analysis were seen, which were further analysed by DNA cloning and sequencing. PCR reactions were performed in 8-fold, pooled and treated with proteinase K. Subsequently, products were ligated into the pGEM T-vector (Promega, Madison, USA) according to the manufacturer's protocol and the clones were sequenced by the dideoxy chain termination method [298].

Radioactive PCR fragment length analysis

The sizes of the PCR fragments comprising the dinucleotide repeat in the promoter region were further analysed on an 8% denaturing polyacrylamide gel by comparison with a sequencing ladder, which was generated using the dideoxy chain termination method [298]. For visualising the PCR products, radioactive α^{32}P-dCTP was added to the PCR reaction. For denaturation of the DNA, the samples were heated for 3 min at 94˚C . Subsequently, 3.5 µl of the samples was loaded on an 8% denaturing polyacrylamide gel and electrophoresed at 60 W during 2 h and developed overnight by autoradiography at -80˚C.

Statistical analysis

Allele frequencies in patient and control groups were compared using a χ^2-test.

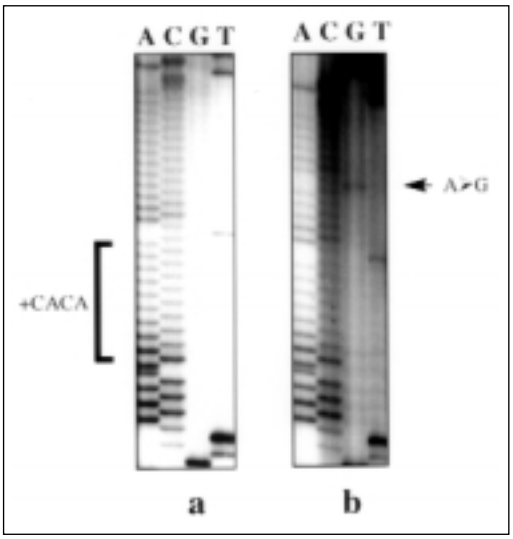

Figure 2. Identification of the different alleles of the imperfect dinucleotide repeat by sequencing. Autoradiographs of sequencing patterns of 2 patients with abnormal shift patterns in the SSCP analysis of the dinucleotide repeat in the 5' upstream region of the SKALP gene *(see Fig 1b). (a)* Patient homozygous for the extra dinucleotide repeats. Two extra CA repeats were seen. *(b)*. Patient with an aberrant SSCP pattern. In the third CA repeat an adenosine to guanosine transition can be seen *(compare with Fig 2a)*.

Results

SSCP and DNA sequence analysis of exons 1 and 2 of the SKALP gene

The human SKALP gene contains 3 exons and 2 introns [299]. The first exon includes the 5'-non-coding region and the signal sequence as well as the first 4 amino acids of the transglutaminase substrate domain of the mature protein. The second exon encodes the rest of the transglutaminase substrate motifs, the proteinase inhibiting domain and contains the first nucleotide of the 3'-noncoding region. The rest of this region, including the polyadenylation signal constitutes the third exon.

SSCP analysis using primer pair 1 revealed sequence variation in exon 1 (Fig. 1a), which suggested that 2 alleles were present with an estimated frequency of the minor allele of 0.095 in the control population. The allele frequencies in the plaque type and pustular psoriasis group were both 0.12 and were not significantly different from the control group. One plaque psoriasis patient was homozygous for the minor allele. From this patient and from a patient who was homozygous for the other allele, the PCR product was cloned and sequenced. The obtained sequence was compared with published nucleotide sequences of the human SKALP gene and cDNA [251,252,256]. At position +43 from the translation start site an adenosine to guanosine transition was found. This mutation results in an amino acid substitution of threonine for alanine in the region coding for the signal peptide. In the SSCP analysis of PCR products from exon 2, using primer pairs 2 and 3, no altered migration patterns were detected.

A dinucleotide repeat polymorphism in the 5'-upstream region

When we sequenced the flanking region of the human SKALP gene, we noted a dinucleotide repeat in the 5'-upstream region of the gene at position -909 from the translation start

site. The form of the repeat is $TCT(CA)_4A(CA)_7CT(CA)_{12}CCC$. PCR reactions on genomic DNA of 48 unrelated individuals using primer pair 4 (Table 1), showed this repeat to be polymorphic. The sizes of the alleles were determined with radioactive PCR fragment analysis revealing a 156 bp PCR product and a 160 bp product. The estimated allele frequencies are 0.135 for the 160 bp allele and 0.865 for the 156 bp in the control group. Mendelian inheritance was observed in one healthy three-generation family. In the plaque psoriasis patient group the allele frequency of the minor allele was 0.12 and in the pustular psoriasis patient group 0.07, which both were not significantly different from the control group. There was one homozygous patient for the 160 bp allele, who had a plaque type psoriasis. From this patient and a control person who was homozygous for the 156 bp allele the PCR products were cloned and sequenced. The 160 bp allele contained two additional CA repeats, resulting in a sequence as follows: $TCT(CA)_4A(CA)_9CT(CA)_{12}CCC$ (Fig. 2a).

Subsequently, SSCP analysis of PCR products from primer pair 4 was performed, which revealed different migration patterns. Two patterns could be contributed to the polymorphism in the repeat and were in concordance with the results of radioactive PCR fragment analysis (Fig. 1b). However, one aberrant pattern was only found in a single patient with plaque type psoriasis (Fig. 1b). From this patient the PCR product was cloned and sequenced. The sequence revealed an adenosine to guanosine transition in the third CA-repeat at position -871 from the translation start site (Fig. 2b).

Discussion

Previous studies revealed decreased protein levels of SKALP in skin extracts of pustular psoriasis patients compared to plaque psoriasis patients [261]. The same observation was made in a patient with impetigo herpetiformis; a pregnancy induced form of generalised pustular psoriasis [300]. These findings made SKALP a candidate gene for pustular forms of psoriasis, and we therefore investigated whether disease-associated sequence variation was present in functionally significant areas of the SKALP gene. In this study an SSCP survey of the SKALP gene in patients with plaque type and pustular psoriasis is presented. In exon 1 a polymorphism was found which results in an amino acid substitution of threonine for alanine in the signal peptide encoding region. Because it implies the change of a relatively hydrophobic amino acid into a hydrophilic amino acid, this could result in a functional change. However, since there was no association between psoriatic patients and the minor allele, we conclude that the decrease in SKALP activity in pustular psoriasis is not caused by or related to this particular polymorphism. The analysis described herein was carried out under conditions designed for optimal detection of mutation. Nonetheless, we cannot exclude the possibility that mutations have been missed, as the detection efficiency of SSCP analysis is approximately 90-95%.

When the flanking sequences of the SKALP gene were analysed we noted a dinucleotide repeat in the promoter region of the gene, which was polymorphic. No association was found between pustular psoriasis and these alleles. We have recently determined multiple

mRNA transcription startsites of the SKALP gene, and we have mapped relevant promoter elements for transcription factor binding (manuscript in preparation). Previously, Zhang et al. sequenced the promotor region and identified several putative transcription factor binding sites [301]. The dinucleotide repeat is situated at pos. -909, and so far does not coincide with the detected regulatory elements. It is therefore unlikely that the mutation found in this repeat in one psoriatic patient (adenosine to guanosine), or the variation in the number of CA repeats will be of functional significance.

In summary, the data presented here strongly suggest that the genetic predisposition to pustular psoriasis is not primarily determined by variation in the SKALP gene. Further, it is unlikely that the observed decrease of SKALP protein levels in the epidermis is based on mutations in the coding regions of the gene. However, because we did not perform SSCP analysis on exon 3, which encodes the 3'-noncoding region, it is still possible that polymorphisms exist in this region that could control translation efficiency. Our present results are in line with our previous finding that *in vivo* the transcript levels of SKALP are equal in pustular and plaque psoriasis patients [261]. Apparently, both are indicating that the decrease of SKALP in pustular psoriasis is due to post-transcriptional events.

Cutaneous stress response pathways

TNF-a and serum induce SKALP gene expression in human keratinocytes by a p38 MAP kinase dependent pathway

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Abstract

Keratinocytes of inflamed epidermis (psoriasis, wound healing) are hyperproliferative and display an abnormal differentiation program. This regenerative differentiation pathway is characterised by the induction of genes that are not expressed by keratinocytes in normal skin, such as the cytokeratins CK6, CK16, CK17, and the proteinase inhibitor SKALP/elafin. Here we have studied the induction and regulation of SKALP expression as a marker for regenerative differentiation in epidermal keratinocytes. Various cytokines and growth factors that are known to be present in psoriatic epidermis were examined for their ability to induce SKALP gene expression in cultured human keratinocytes. Tumour necrosis factor-alpha (TNF-α) and serum were found to be potent inducers of SKALP expression both at the mRNA and protein level. To examine a possible role of the MAP kinase cascade in SKALP expression we have investigated the activation of the different members of the MAP kinase family, using phosphorylation-specific antisera on western blots. Detectable levels of activated (phosphorylated) components of all three MAP kinase pathways were found in undifferentiated and differentiated keratinocytes. When keratinocytes were induced to undergo differentiation, the phosphorylation levels of ERK1/2 and c-jun, a major target of JNK/SAPK, remained nearly constant, whereas the phosphorylation levels of p38 showed a minor increase concomitantly with induction of normal and regenerative differentiation. However, SB202190 or SB203580, two specific p38 MAP kinase inhibitors could almost completely block the induction of SKALP expression by TNF-α and serum. These results suggest that in keratinocytes, p38 activation is a necessary, albeit not sufficient step in the induction of SKALP gene expression. These findings could be relevant for understanding the mechanisms that are involved in normal and disturbed epidermal differentiation.

Introduction

In normal skin the epidermal keratinocytes follow a highly co-ordinated pathway of proliferation and terminal differentiation leading to the formation of the horny layer which consists of mature corneocytes [260]. However, keratinocytes are also capable of following an alternative differentiation route generally known as regenerative differentiation or regenerative maturation [6,7]. Regenerative differentiation is a physiological adaptation program of keratinocytes to a disturbance of epidermal homeostasis, like skin barrier disruption and ultraviolet light (UV) irradiation [202,302,303]. Also in several pathological skin conditions, e.g. psoriasis and squamous cell carcinoma, keratinocytes follow this abnormal differentiation pathway [10,245]. Regenerative differentiation is in many ways distinct from the differentiation program found in normal, healthy human skin. A number of proteins normally found in epidermis are overexpressed. These include proteins that are involved in the formation of the corneocyte such as involucrin and transglutaminase-1. Normally the expression of these proteins is restricted to the *stratum granulosum* but during regenerative differentiation these proteins are also expressed in the *stratum spinosum* [304]. An even

more interesting feature, from a mechanistic point of view, is the expression of proteins that are normally absent in human epidermis but are now highly expressed. Well known examples are the cytokeratins (CK) 6, 16 and 17 [12] and the epithelial proteinase inhibitor skin-derived antileukoproteinase (SKALP), also known as elafin, which is a member of the Trappin gene family that we have recently described [305]. The expression pattern of CK6/16/17 and SKALP is tightly associated with the keratinocyte phenotype of regenerative differentiation *in vivo*. We hypothesise that knowledge of the regulatory factors and pathways that are involved in the expression of these genes in keratinocytes will contribute to elucidate the processes that are involved in the transition between epidermal differentiation programs, as seen in psoriasis, wound healing and some forms of cutaneous neoplasia.

Epidermal keratinocytes are in vivo regularly exposed to stimuli such as solar UV irradiation, loss of barrier function and inflammation. Studies in many cell types (mostly *in vitro*) have demonstrated that stimuli such as UV radiation, osmotic stress and inflammatory cytokines exert their effects on the cell by the activation of the so-called cellular stress response pathway. The cellular stress response pathway is a conserved eukaryotic signal transduction route that enables the cell to adapt to damaging environmental stimuli. Through a tightly regulated cascade, a family of protein kinases known as the Mitogen Activated Protein kinases (MAP kinases) can become active upon phosphorylation. In an activated state, these MAP kinases can phosphorylate, and thereby activate, a variety of transcriptional regulatory factors. In this way, by changing the cells transcription factor repertoire, activation of the MAP kinase pathway decides on gene expression and thereby cell fate (apoptosis versus survival) [306,307]. Recently, the relevance of the MAP kinase members in UVB induced gene expression in human skin in vivo has been described [130,133,308]. In addition to its role in stress-induced gene expression, it was recently shown that regulation of the involucrin promoter, a protein associated with normal differentiation, is dependent on activity of MAP kinase family members [181]. The role of the MAP kinase cascade in the regulation of regenerative epidermal differentiation has not been studied sofar.

Because SKALP is known to be expressed in vivo when keratinocytes are exposed to inflammatory cytokines (psoriasis [277,309], wound healing [310]) and osmotic stress (skin barrier disruption by tape stripping [202] or detergent [205]) we have investigated regulation of SKALP gene expression by keratinocytes as a paradigm for the abnormal regenerative differentiation program. To study the effects of selected cytokines we used a recently described submerged keratinocyte model which allows controlled induction of both normal differentiation (expression of involucrin, transglutaminase, CK1/10) and regenerative differentiation (expression of SKALP, psoriasin) [77,294]. Furthermore we have investigated the phosphorylation, and thereby the activation, of the three main MAP kinases, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAP kinase during induction of normal and regenerative differentiation. Using pharmacological inhibitors we found that p38 activity is necessary for the induction of SKALP gene expression following stimulation by TNF-α or serum.

Materials and methods

Cell cultures
Keratinocytes were seeded in 6 well culture dishes and cultured until confluence in keratinocyte growth medium (KGM) as described before [77]. KGM was composed of keratinocyte basal medium (KBM (Biowhittaker, Walkersville, Maryland, USA); 0.15 mM Calcium), supplemented with ethanolamine (0.1 mM) (Sigma, St.Louis, USA), phosphoethanolamine (0.1 mM) (Sigma, St.Louis, USA), bovine pituitary extract (BPE; 0.4 $\%^{v}/_{v}$) (Biowhittaker, Walkersville, Maryland, USA), insulin (5 μg/ml), (Sigma, St. Louis, USA), hydrocortisone (0.5 μg/ml) (Collaborative Research Inc.), recombinant mouse Epidermal Growth Factor (EGF; 10 ng/ml) (Sigma, St. Louis, USA), penicillin (100 U/ml) (Gibco, Breda, the Netherlands) and streptomycin (100 μg/ml) (Gibco, Breda, the Netherlands). At confluence the cells were switched to one of the test media, subsequently the growth factor or cytokine that was to be tested was added to the culture medium in different concentrations. Induction of keratinocyte differentiation was established by switching the cells at confluence either to KGM supplemented with 5 $\%^{v}/_{v}$ FCS (KGM/FCS) (FCS from Seralab, Nistelrode, the Netherlands) or to KGM depleted of growth factors (BPE, Insulin and EGF) and hydrocortisone (KGM/-gf) [265]. Two different p38 inhibitors were used, SB203580 (a kind gift of Dr. Griswold from SmithKline Beecham, PA, USA) and SB202190 (Calbiochem, CA, USA). The inhibitors were dissolved in DMSO and added in different concentrations to the cultured cells one hour prior to the addition of the differentiation stimulus. Different concentrations of DMSO were tested for the effect on the cultured cells. The cytokines and growth factors that were tested were IL-1α, IL-2, and IL-10 (a kind gift from Dr. L. Joosten, Dept. of Rheumatology, University Hospital Nijmegen), IL-4 (Schering & Plough, Amstelveen, The Netherlands, for research purposes only), IL-6 (a kind gift from Dr. L. Aarden, Central Laboratory for Bloodtransfusion, Amsterdam), IL-8, TGF-α, TGF-β, and IFN-γ (R&D systems, Abingdon, United Kingdom), PDGF, KGF, and TNF-α (Boehringer Mannheim, Mannheim, Germany). At different time points after the addition of stimulus, the cell culture medium was collected and the cells were lysed either in RNAse-all for RNA isolation or in SDS-PAGE sample buffer (recipe see section western blotting) for western blot analysis.

Enzyme-linked immunosorbent assay (ELISA) for measurement of SKALP/elafin levels
SKALP/elafin concentrations were measured in culture supernatants using a sandwich-type ELISA. In brief, samples were diluted (1:40, 1:60, 1:80) in 0.1 M Tris, 0.1% Tween-20, 1% BSA (ICN Biomedicals, Aurora, Ohio, USA). Microtiter plates (96 flat bottom wells) were coated overnight with goat anti-SKALP/elafin antiserum. After washing of the plate with PBS/0.05% Tween-20, microtiter plates were blocked and probed with the test samples for 2 hrs at 37°C. Subsequently, rabbit anti-SKALP/elafin antiserum with 2.5% normal goat serum (Vector Laboratories, Burlingame, USA) was added and the plates were incubated during 60 minutes at 37°C. Subsequently the microtiterplates were incubated with peroxidase-conjugated swine-anti-rabbit IgG (DAKO, Glostrup, Danmark) with 2.5% normal goat serum during 60 minutes at 37°C. Finally o-phenylenediamine dihydrochlo-

ride (OPD) (Pierce, Rockford, Illinois, USA) was added as chromogenic substrate for 15 minutes at RT. This enzyme reaction was stopped by the addition of 4 M H_2SO_4. Absorbencies were measured at 492 nm and at 655 nm. Human recombinant SKALP/elafin in PBS with 0.1% BSA was used as a standard: a calibration curve was made, using recombinant SKALP in the range of 0.5 - 120 ng/ml. The SKALP/elafin concentrations in the test samples were read from this curve. The SKALP/elafin concentrations given in ng secreted SKALP/elafin/ml culture medium.

RNA-isolation and Northern blot analysis

Cells from which total RNA was to be isolated were lysed in 1 ml RNase-All (2.1 M Guanidine-thiocyanate (Research Organics Inc., Cleveland USA), 8.5 mM N-lauroylsarcosine (Sigma, St. Louis, USA), 12.5 mM NaAc pH5.2, 0.35 $\%^{v}/_{v}$ β-mercapto-ethanol (Merck, Darmstadt, Germany) and 50 $\%^{v}/_{v}$ Tris-saturated biophenol pH8.0 (Biosolve, Amsterdam, the Netherlands)), after lysis 100 µl chloroform was added. The samples were centrifuged for 15 minutes (13000 rpm, 4˚C). The aqueous phase was precipitated and the pellet was washed and dried. This RNA pellet was resuspended in 150 µl NSE (50 mM NaAc, 0.2 % SDS and 2 mM EDTA) and 562.5 µl 100% ethanol was added. Samples were spectrophotometrically quantitated and equal amounts were separated overnight on an agarose gel. The samples were blotted on positively charged nylon and subsequently used in hybridisation experiments. Probes were used against SKALP/elafin, CK1 and for normalisation of the amount of RNA loaded on the gel a probe against the ribosomal protein human Acidic Ribosomal Phosphoprotein (hARP) was used [311]. Autoradiography was performed using Kodak X-Omat X-ray films and Kodak BiomaxMS X-ray films. Autoradiograms were quantitatively analysed using the “Imagemaster 1D elite” image analysis software package (distributed by Hoefer Pharmacia Biotech, Upsala, Sweden).

Western blotting

Using the phosphospecific antibodies kits supplied by New England Biolabs (Beverly, MA, USA) we performed Western blotting and protein detection according to the protocol of the manufacturer. In short, cultured keratinocytes were lysed in sample buffer ((approximately 5000 cells per µl) 62.5 mM Tris pH6.8, 10 % glycerol, 0.1 % bromphenol blue, 2 % SDS, 50 mM DTE). Cell lysates were sonified two times 10 sec. and subsequently incubated for 3 min. at 100˚C. Together with caleidoscopic and biotinylated markers, samples were run on a 10 % SDS PAGE gel (biorad) in Tris-Glycine-SDS buffer (Biorad) at 175 volts. Gels were electro-blotted on PVDF blotting paper (Biorad) in Tris-Glycine-SDS buffer supplemented with 20 % methanol at 400 mA for 35 min. After blocking using Blotto (Pierce, Rockford, Illinois, USA), the blots were incubated in the primary antibody solution (100 mM Tris pH7.5, 0.9 % NaCl, 5 % BSA, 0.1 % Tween-20, 0,1 % NaN_3) in which the phosphospecific antibody (either directed against c-jun phosphorylated at serine-73 site (New England Biolabs), against p38, dually phosphorylated both at the threonine-180 and the tyrosine-182 site (New England Biolabs) or against tyrosine phosphorylated ERK1/2 (New England Biolabs)) was diluted 1:1000. Blots were incubated in this solution for 2 hrs. at room temperature. After washing the blots in TBS (100 mM Tris,

0.9 % NaCl) the blots were incubated for 60 min. at room temperature in Blotto in which the secondary antibodies (against biotinylated marker and against primary antibody) were diluted 2000 times. Subsequently the blots were washed in TBS and incubated in chemiluminescent LumiGlo (New England Biolabs) reagent for 1 min. Finally the blots were packed in food wrap and several exposures were made on Kodak X-Omat X-ray films. For quantification the Lumi-Imager system (Boehringer) was used.

In vitro p38 MAP kinase assay
The in vitro p38 MAP kinase assay was performed according to the procedure provided by the manufacturer (New England Biolabs, Beverly, MA, USA). In short, approximately $2x10^6$ cells were lysed and total p38 was immunoprecipitated. The resulting imunoprecipitate was incubated with GST-ATF-2 fusion protein in the presence of ATP. Phosphorylation of ATF-2 at Thr71 was measured by western blotting using a phospospecific (Thr71) antibody. The same antibody was used to detect phosphorylated endogenous ATF-2 in keratinocyte extracts.

Results

SKALP expression in human keratinocytes is strongly induced by Tumour Necrosis Factor-α
We tested a number of cytokines and growth factors that are known to be present in inflamed skin and hyperproliferative skin for their ability to induce SKALP expression in normal human keratinocytes *in vitro*. These stimuli (see materials and methods) were tested in a submerged culture model that allows normal differentiation of the keratinocytes, as we have described previously [77,294]. This model uses growth factor depletion of a culture that was first grown to confluence in KGM. At 48 hrs after culturing in growth factor depleted medium (KGM/-gf) involucrin, transglutaminase-1 and cytokeratins 1/10 are highly expressed, while SKALP is not expressed. As a positive control for induction of regenerative differentiation we used KGM supplemented with FCS (KGM/FCS) which was shown previously to induce expression of SKALP and psoriasin, both markers for regenerative differentiation. Of all stimuli we tested, only TNF-α induced a strong, dose-dependent increase of SKALP mRNA of similar magnitude as FCS, as assessed by northern blot analysis (figure 1). IFN-γ and TGF-α induced a small increase in SKALP mRNA, whereas all other cytokines and growth factors were negative (data not shown). Induction of SKALP was found both at intermediate (0.15 mM) and high (1.85 mM) Ca^{2+}. SKALP expression induced by TNF-α was also found at the protein level as assessed by ELISA (figure 2). SKALP expression reached a plateau between 5 and 20 ng/ml TNF-α; therefore, a concentration of 20 ng/ml was used in further studies.
In a time course experiment TNF-α rapidly induced SKALP expression both at the mRNA and protein level. Already 4 hrs after stimulation the mRNA signal was higher than background, and the peak of mRNA accumulation in keratinocytes could be observed at 48 hrs after TNF-α stimulation (figure 3), whereas SKALP protein accumulation in the cultured medium continued up to 96 hrs after TNF-α stimulation (figure 4). For comparison, kera-

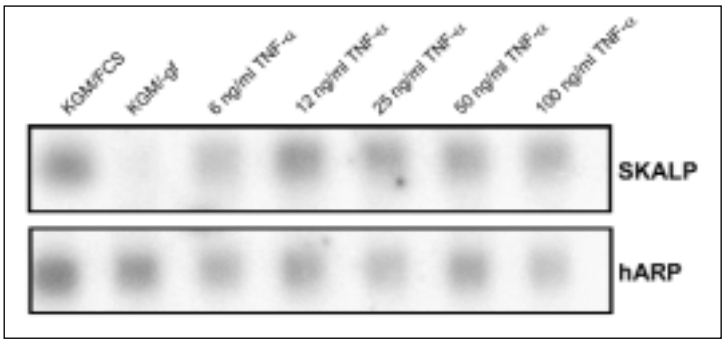

Figure 1. Northern blot analysis of SKALP expression by cultured human keratinocytes incubated with different concentrations of TNF-α. The concentrations of TNF-α (ng/ml) are indicated above the lanes. Confluent layers of keratinocytes were induced to differentiate in KGM depleted of growth factors for 48 hours. Subsequently the medium was refreshed, different concentrations of TNF-α were added and the cells were cultured for another 48 hours. Addition of TNF-α results in a concentration dependent expression of the SKALP gene that reaches its maximum level around $12^1/_2$ ng/ml TNF-α. Foetal calf serum (FCS) a known inducer of SKALP gene expression was used as a positive control (first lane). The human Acidic Ribosomal Phosphoprotein PO (hARP) was used for control hybridisation to check for equal RNA loading (bottom row).

tinocyte cultures that were induced with 5% FCS showed a peak in SKALP mRNA and protein accumulation after 48 h (figure 4).

Phosphorylation levels of MAP kinase family members remain nearly constant regardless of keratinocyte differentiation status.

The phosphorylation status of ERK 1/2, p38 and c-jun (a major substrate of the JNK MAP kinase) during induction of regenerative differentiation was examined, using phosphorylation-specific antisera on western blots. The levels of phosphorylated (activated) protein were compared to the levels of total (phosphorylated plus unphosphorylated) protein as a reference (see figure 5).

Using an antiserum directed against the phosphorylated serine-73 epitope, it is shown that there is no significant change in c-jun phosphorylation levels during induction of differentiation by FCS (figure 5, panel A, lanes 19-24). Phosphorylated c-jun is already present, albeit at low levels, in undifferentiated keratinocytes cultured in KGM (figure 5 panel A, lanes 13-18). When TNF-α was added to keratinocytes that were already differentiated by growth factor depletion (KGM/-gf), a small, immediate increase in c-jun phosphorylation was seen (up to 4 hrs (figure 5, panel A, lanes 7-10)) which returned to control levels at later time points compared to the KGM/-gf cultures.

The MAP kinase family members ERK1/2 (also known as p42/p44) are activated by phosphorylation of a tyrosine and a threonine residue. Our experiments were performed using a polyclonal antibody against tyrosine phosphorylated ERK1/2 (recognising both phosphorylated ERK1 (p44) and ERK2 (p42)). ERK1/2 were found to be phosphorylated both in undifferentiated keratinocytes (cultured in KGM) and in keratinocytes induced to

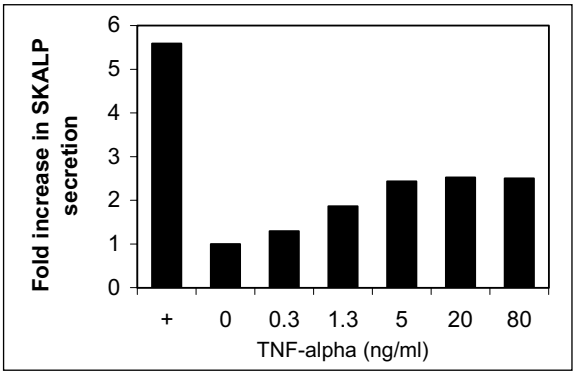

Figure 2. Analysis of the induction of SKALP secretion by keratinocytes incubated with different concentrations of TNF-α. The concentrations of TNF-α (ng/ml) are indicated under the columns. Confluent layers of keratinocytes were induced to differentiate in KGM depleted of growth factors for 48 hours. Subsequently the medium was refreshed, different concentrations of TNF-α were added and cells were cultured for another 48 hours. The concentration SKALP secreted in the culture medium by the keratinocytes was determined using a sandwich-type ELISA. The concentration of SKALP secreted in the medium is given as a fold-increase relative to unstimulated cells (which was 44 ng/ml). Foetal calf serum (FCS) a known inducer of SKALP gene expression was used as a positive control (first column).

differentiate with FCS (figure 5, panel B). An increase of phosphorylated ERK 1/2 is seen, during differentiation. However, the relative phosphorylation levels remain more or less constant as the levels of total ERK 1/2 rise concomitantly. No gross changes in relative phosphorylation levels were noted in normally differentiated cultures that were stimulated with TNF-α relative to the control culture (KGM/-gf).

The MAP kinase family member p38 can become active upon dual phosphorylation of a tyrosine residue at position 182 and a threonine residue at position 180. We have used an antiserum that recognises dually phosphorylated p38 (figure 5, panel C). Undifferentiated keratinocytes contain detectable levels of dually phosphorylated p38 at time zero (KGM). These levels remain low or decrease even further, up to 48 hrs after confluence in KGM. The level of phosphorylated p38 remains nearly constant during the first hours after the induction of differentiation by FCS. Twenty-four and 48 hrs after induction there is a small increase in the p38 phosphorylation levels in the keratinocytes. Quantitative image analysis of the blots indicated a 2.5 fold increase of p38 phosphorylation levels at 24-48 hours compared to undifferentiated cultures in KGM. When keratinocytes were induced to differentiate by growth factor depletion (KGM/-gf), which leads to expression of the normal differentiation phenotype, p38 phosphorylation levels were also found to be increased at 48 hrs after induction (not shown here). Addition of TNF-α to a normally differentiated culture only induced a marginal additional increase in p38 phosphorylation at 24-48 hrs compared to the control culture (KGM/-gf). These findings indicate that although there is a mild increase in p38 phosphorylation during differentiation, the increase in p38 phosphorylation levels is not specific for the normal or regenerative differentiation phenotype.

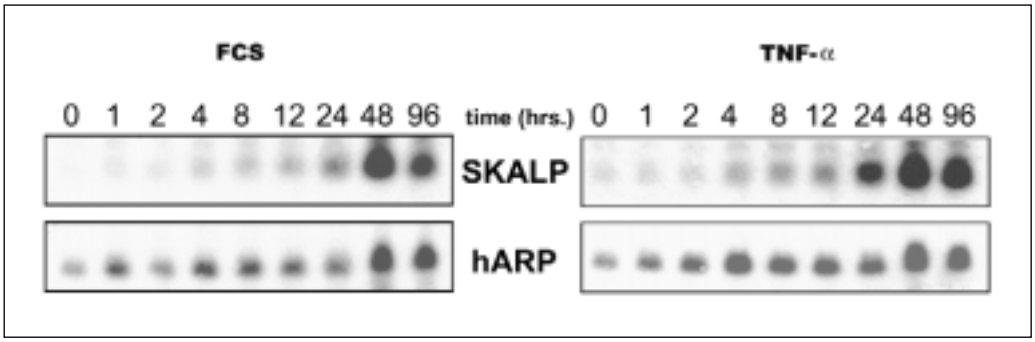

Figure 3. Northern blot analysis of the time course of SKALP expression induced by TNF-α or FCS. The time (in hrs) after the inducer (TNF-α or FCS) was added is indicated above the lanes. Keratinocytes were grown to confluence in KGM. Differentiation was induced by the addition of 25 ng/ml TNF-α in the presence of 1.85 mM Ca^{++} or by the addition of 5 % FCS. At regular time intervals the culture medium was collected and the cells were harvested. Approximately eight hours after TNF-α is added to the cells, SKALP mRNA levels begin to rise. This rise continues until at least 48 hours after the induction of SKALP expression by TNF-α. At the mRNA level the induction of SKALP expression by FCS reaches a peak 48 hrs after FCS was added to the cells. The human Acidic Ribosomal Phosphoprotein PO (hARP) was used for control hybridisation to check for equal RNA loading. At the bottom row the relative expression levels of SKALP are given as a fold increase in the SKALP/hARP ratio relative to the situation at t=0 (assigned the value 1).

This finding was further illustrated by measuring p38 MAPkinase activity in keratinocyte extracts using an vitro kinase assay (figure 6) with recombinant ATF-2 as a substrate. Similar levels of kinase activity were found both in normally differentiated keratinocytes that do not express SKALP (KGM/-gf (figure 6, panel A, lane 2)) and in keratinocytes that have undergone regenerative differentiation and express SKALP (figure 6, panel A, lanes 1 (KGM/FCS) and 3 (KGM/TNF)). This finding correlates with the level of phosphorylated p38 under these culture conditions (panel B). As a reference the level of total p38 is depicted in panel C. These levels of endogenous p38 expression, phosphorylation and activity, however, do not correlate with the presence of phosphorylated, endogenous ATF-2, a putative major downstream target for p38 (panel D).

Inhibition of p38 activity in keratinocytes abolishes SKALP expression induced by FCS and TNF-α

To investigate whether p38 activity, present in differentiated keratinocytes, is required for SKALP gene expression following stimulation by FCS or TNF-α, we tested the effects of the p38 specific inhibitors SB203580 and SB202190. These pyridinyl-imidazole compounds were previously shown to be very potent, highly specific inhibitors for several p38 MAPkinase isoforms that showed no appreciable activity towards other kinases. SB202190 almost completely blocked the induction of SKALP gene expression, both at the protein and mRNA level (figure 7 and 8). Similar results were obtained for SB203580 (data not shown). The expression level of the household gene hARP was unaffected by the p38 inhi-

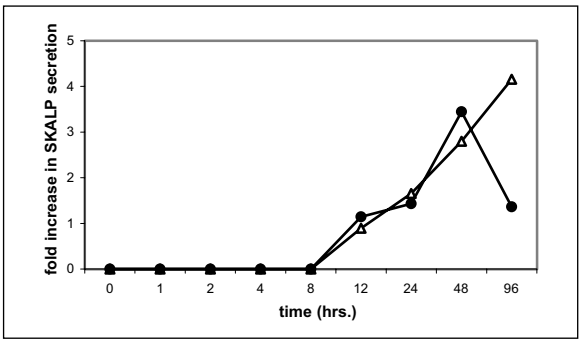

Figure 4. Time course of SKALP secretion by cultured human keratinocytes after induction by TNF-α or FCS. Keratinocytes were grown to confluence in KGM. Differentiation was induced by the addition of 25 ng/ml TNF-α in the presence of 1.85 mM Ca^{++} or by the addition of 5 % FCS. At regular time intervals (X-axis) the culture medium was collected and the cells were harvested. The SKALP concentration in the collected culture media was measured. The SKALP levels are given as a fold-increase relative to unstimulated cells. Approximately eight hours after the addition of TNF-α, massive induction of SKALP expression can be measured (open triangles). SKALP secretion continues even after 96 hours. After induction by FCS the keratinocytes express SKALP with a peak expression after 48 hours (closed circles). Note that 48 hours after the induction was induced the cells were given fresh culture medium explaining the drop in SKALP levels in the medium at time point 96 hours after FCS addition.

bitors. No apparent cytotoxicity was found either by morphological examination or LDH release, in the concentration range that induced inhibition of SKALP expression (not shown).

Discussion

A distinct feature of abnormal epidermal differentiation (as seen in psoriasis, after UV irradiation, during wound healing and in epithelial neoplasia) is the *de novo* synthesis of a set of proteins which are absent in normal epidermis. These include the cytoskeletal proteins CK6, CK16 and CK17 and the proteinase inhibitor SKALP/elafin [12,13,77,312]. Although these proteins are not necessarily relevant for the pathogenesis of these skin conditions *per se*, their expression is tightly associated with the alteration of keratinocyte differentiation in the context of hyperproliferation. We hypothesise that elucidation of signalling pathways and factors that are relevant for regulation of these *de novo* expressed genes, could contribute to understanding the general mechanism of altered epidermal differentiation as seen in e.g. psoriasis. In this study we have used the induction and regulation of SKALP gene expression as a paradigm for the induction and regulation of regenerative differentiation. We have previously shown that SKALP is highly expressed in psoriatic lesional skin, and that it is induced upon external stress such as osmotic stress (tape stripping) [246], chemical stress (low doses of detergent) [205] and UV-irradiation (Pfundt *et al*, in prepara-

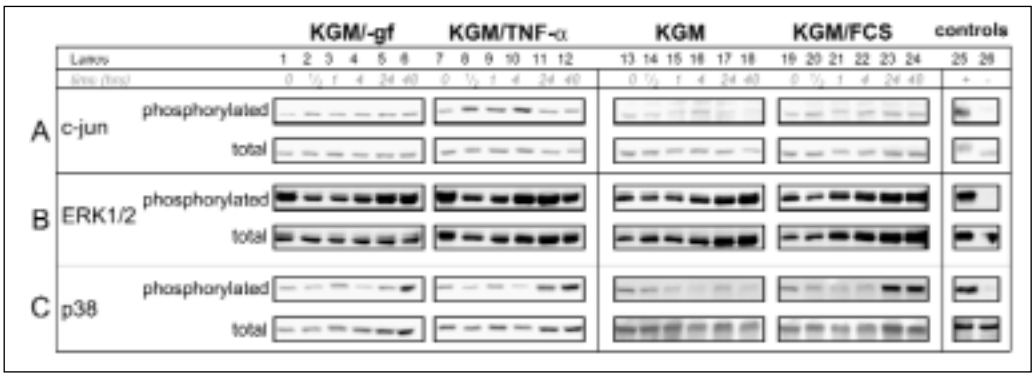

Figure 5. Analysis of the phosphorylation status of proteins of the stress response pathway (c-jun, ERK1/2 and p38) by western blot analysis. Confluent cells were cultured for another 48 hours in either KGM before FCS stimulation (KGM/FCS) or in KGM depleted of growth factors (KGM/-gf) before TNF-α stimulation (KGM/TNF). Subsequently the culture medium was replaced by fresh medium with (FCS or TNF-α) or without (for control analysis) stimulus. At regular time intervals (as indicated above the blots and under the lane numbers) the cells were harvested in sample buffer and stored for western analysis. The western blots were stained for total and phosphorylated c-jun *(panel A)*, ERK1/2 *(panel B)* and p38 *(panel C)*. Note that only for p38, changes in relative phosphorylation levels were found upon FCS or TNF-α stimulation. Quantitative analysis indicated a 2.5 fold increase 48 hours after FCS stimulation (panel C, lane 24) compared to unstimulated cells (panel C, lane 18). For TNF-α quantification revealed a slight increase in relative p38 phosphorylation (1.5 fold) 48 hours after TNF-α stimulation (panel C, lane 12) compared to unstimulated cells (panel C, lane 6). The specificity of the antibodies was checked by using control cellysates (control) containing either phosphorylated forms of c-jun, ERK1/2 and p38 (indicated by +) or unphosphorylated forms of these proteins (indicated by -).

tion). Recently we have shown that SKALP expression can be induced in submerged culture models for keratinocyte differentiation, which are used here to test the effect of cytokines and growth factors that are relevant for skin conditions such as psoriasis and wound healing [77]. The finding that TNF-α is a potent inducer of SKALP gene expression is in agreement with the notion that in lesional psoriatic skin the infiltrating T-lymphocytes are of the Th_1 phenotype (high levels of IL-2, IFN-γ and TNF-α) [313,314]. Interestingly, IFN-γ and TNF-α both have a growth inhibitory effect on keratinocytes in vitro, rather than promoting hyperproliferation which is characteristic for the regenerative differentiation phenotype [315,316]. These results imply that the mere exposure of keratinocytes to TNF-α or IFN-γ can not be the sole trigger to induce the complete phenotype of keratinocytes found in hyperproliferative epidermis.

Because in vivo we have found that stress factors, as mentioned above, can induce SKALP expression we asked whether cellular stress response pathways would be involved in regulation of SKALP expression. The activity of these proteins in many cell types including keratinocytes has been described in detail. The major role of these proteins is to relay external stress signals to the nucleus. Through multiple phosphorylation levels this pathway changes the cellular transcription factor repertoire and thereby regulates gene expres-

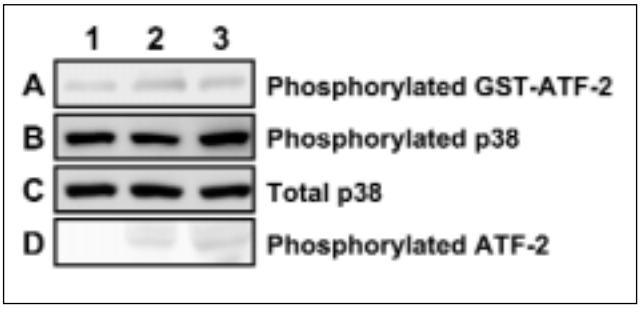

Figure 6. Analysis of p38 activity by and *in vitro* kinase assay. Confluent cultures of keratinocytes were allowed to differentiate for 48 hours in different media, either with the expression of SKALP i.e. KGM/FCS (lanes 1) and KGM/tnf (lanes 3) or without the expression of SKALP i.e. KGM/-gf (lanes 2). The cells were harvested either in lysis buffer for kinase assays or in sample buffer for western blot analysis. An in vitro kinase assay was performed on the samples to measure p38 MAP kinase activity (panel A). In addition the western blot samples were stained for phosphorylated p38 (panel B), total p38 (panel C) and phosphorylated endogenous ATF-2 (panel D).

sion patterns of the cell or determines the fate of the affected cells (survival versus apoptosis) [317-319]. The ERK family members seem to act as regulators of cell proliferation and have repressive effects on cell differentiation [320-323]. Here we have found that phosphorylated ERK 1/2 is present in cultured keratinocytes. Induction of differentiation did not appreciably alter the relative phosphorylation levels of ERK 1/2. We could not examine the effect of ERK 1/2 inhibition in this system because the addition of a pharmacological inhibitor of MEK1 (PD98059), which would prevent ERK 1/2 phosphorylation resulted in cell death even at very low concentrations (R. Pfundt unpublished results). Taken this into account it appears that ERK1/2 is essential for keratinocyte proliferation and survival and could play a role in the hyperproliferative component of the regenerative differentiation phenotype.

Phosphorylated c-jun, which is major substrate of the JNK MAP kinase family member [306,324] can homodimerise or form heterodimers with several other proteins leading to the formation of AP-1 transcription factor complexes. It has been well documented that AP-1 transcription factor complexes play a major role in the regulation of normal keratinocyte differentiation [325-328]. In vivo, c-jun was found to be predominantly localised in the differentiated compartment [326,329]. In our cultures phosphorylated c-jun is found both in undifferentiated and differentiated keratinocytes. No strong increase in c-jun phosphorylation was noted during induction of differentiation with FCS. Addition of TNF-α to a normally differentiated culture induced a rapid minor increase in c-jun phosphorylation. It remains to be investigated if phosphorylated c-jun is directly involved in SKALP gene transcription, as was suggested by promoter studies in breast tumour cells [330].

In many cell types p38 MAP kinase is known to be activated shortly (30-60 minutes) after stimulation e.g. with UV or TNF-α [142,331,332]. This is in contrast with our experiments

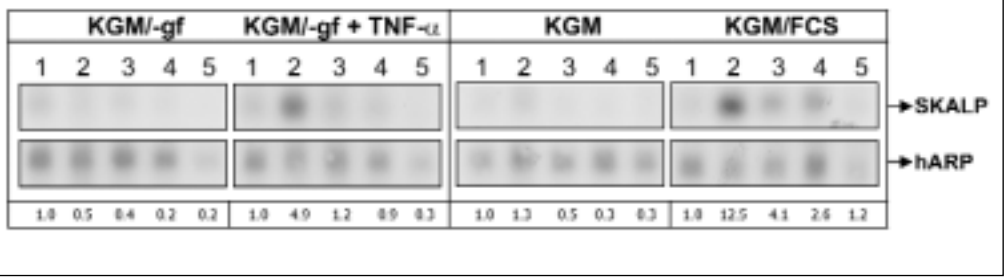

Figure 7. Northern blot analysis of the effect of different concentrations p38 MAP kinase inhibitor SB202190 on the induction of SKALP gene expression. The cells were incubated with p38 inhibitor one hour prior to stimulation by either FCS or TNF-α; RNA was isolated 48 hours after stimulation. The culture conditions are indicated in the boxes above the blots. The concentrations SB202190 given to the cells were 0 μM (lanes 2), 3.8 μM (lanes 3), 7.5 μM (lanes 4) and 15 μM (lanes 5). Lanes 1 contain RNA of cells that were not stimulated (note that in the case of KGM/gf and KGM these conditions are identical to those of lane 2). The human Acidic Ribosomal Phosphoprotein PO (hARP) was used for control hybridisation to check for equal RNA loading (Bottom row). Lanes 2-5 of FCS and TNF-α stimulated cells show a dose dependent decrease of SKALP mRNA expression by SB202190. At the bottom row the relative expression levels of SKALP are given as a fold increase in the SKALP/hARP ratio relative to the situation at t=0 of each culture condition (lanes 1, assigned the value 1).

where we did not find significant increases in p38 phosphorylation shortly after TNF-α or serum stimulation, but rather a gradual small increase in p38 phosphorylation levels during induction of differentiation. A recent paper describes the involvement of p38 MAP kinase in the regulation of involucrin gene expression, an important marker for the normal terminal differentiation route in keratinocytes [181]. These findings couple normal keratinocyte differentiation with p38 activity. However the family of p38 MAP kinases consists of at least 5 isoforms (i.e. p38, p38-β, p38-β2, p38-γ and p38-δ) [137]. With respect to substrate specificity and sensitivity to inhibition by the pyridinyl-imidazoles, SB203580 and SB202190, there is some controversy in the literature. Some authors demonstrate inhibitory activity against the α-, β-, and γ-isoforms [136] whereas others show that the activity of the β1- and γ-isoforms is not affected [137]. It is however clear that the pyridinyl-imidazole compounds are p38 specific and show little or no inhibitory effects on the ERK or JNK MAP kinase family members [333,334]. Here we used the selective p38 inhibitors SB202190 and SB203580 to show that both FCS and TNF-α induced SKALP expression are p38 dependent. Hence, the activity of piridinyl imidazole sensitive p38 isoforms seems essential. However since we can not demonstrate a difference in p38 phosphorylation or activity levels between cells that do or do not express SKALP, we speculate that the differential expression is regulated at a level downstream of p38, or by a p38 isoform that is inhibited by SB202190 but is not recognised by the antibodies used in the western blot analysis and kinase assay. With respect to p38 activity it remains unclear what downstream effects and substrates play a role in the induction of SKALP gene expression in our *in vitro* models for epidermal differentiation. It is, however, remarkable that, in the samples used for the *in*

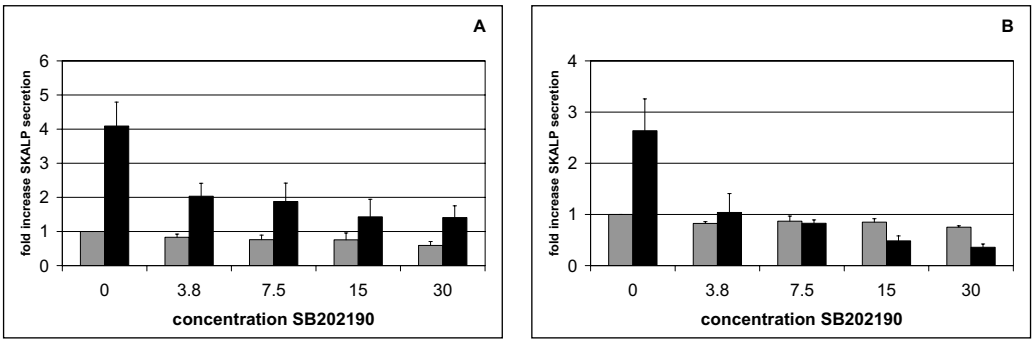

Figure 8. The effect of different concentrations p38 inhibitor SB202190 on the secretion of SKALP by keratinocytes. The cells were incubated with p38 inhibitor one hour prior to stimulation by either FCS or TNF-α, SKALP protein concentrations in the culture media were measured 48 hours after stimulation. SKALP production is given as folds increase relative to unstimulated keratinocytes. Error bars represent the standard error of the mean (SEM) based on three independent experiments. Black columns represent the relative increase in SKALP secretion by the keratinocytes that were stimulated by either FCS (panel A) or TNF-α (panel B). Grey columns represent the relative increase in SKALP secretion in the control conditions, i.e. KGM (panel A) or KGM/gf (panel B). Note that both FCS and TNF-α induced SKALP expression show a dose dependent decrease by the addition of the p38 inhibitor SB202190 ($p<0.05$, ANOVA).

vitro kinase assay, the levels of phosphorylated, endogenous ATF-2, a presumed major downstream target for p38, do not correlate with the p38 activity found. Therefore, findings using a substrate in an in vitro kinase assay, should be interpreted with some caution, and do not necessarily reflect the kinase action within the cell. These findings, and the fact that p38 could be involved in transcription of the involucrin gene, suggest that p38 is involved both in normal and regenerative differentiation, but could confer its activity through different substrates. Further experiments are required to establish if p38 activity is necessary for other aspects of epidermal differentiation as well. Preliminary experiments indicate that not all markers for keratinocyte differentiation are equally affected by p38 inhibition (Pfundt et al, unpublished results). Because a number of antipsoriatic agents (e.g. retinoids, vitamin D derivatives) are known to alter epidermal differentiation pathways and can suppress SKALP expression [335,336], this raises the possibility that p38 inhibitors could be effective for therapeutic applications. It would be interesting to investigate whether the p38 pathway is effective in suppressing other features of the regenerative keratinocyte phenotype as seen in psoriasis, such as hyperproliferation and increased cytokine production. Based on the fact that p38 was initially discovered as an inducer of cytokine production [140] and its effect on keratinocyte differentiation, we would hypothesise that p38 MAP kinase inhibitors could be potentially useful as a new generation of antipsoriatic agents.

Ultraviolet-B irradiation of human epidermis induces activation of cellular stress response pathways and expression of genes involved in cell protection and survival.

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Abstract

Recently, it was shown that ultraviolet B irradiation of human skin leads to activation of cellular stress response pathways that induce expression of metalloproteinases. These findings, which offer an explanation at the molecular level for UVB-induced photodamage to dermal collagen and elastin, prompted us to investigate if UVB could also induce protective mechanisms in human skin in vivo. Here we describe the response of human epidermis and cultured human keratinocytes to UVB irradiation, with respect to the induction of genes that are associated with host protection against tissue damage and infection, in the acute phase of cutaneous inflammation. Two of these genes, SKALP/elafin and SLPI both serine proteinase inhibitors and antimicrobial proteins were found to be highly expressed following UVB irradiation of human skin in vivo. Expression of these genes by differentiated keratinocytes was preceded by activation of cellular stress response pathways such as phosphorylation of c-jun and p38 MAP kinase as determined in situ by immunohistochemistry using phosphorylation-specific antisera. In vitro studies with cultured keratinocytes indicated that SKALP and SLPI expression could be induced by TNF-α or serum, and that this process was dependent on p38 MAP kinase. Although UVB irradiation of cultured keratinocytes was followed by a huge increase in p38 activation, this was not sufficient to increase SKALP or SLPI expression levels suggesting that induction of these genes by UVB in vivo require additional signals. More strikingly, inhibition of p38 MAP kinase activity following UVB irradiation resulted in massive cell death, suggesting that p38 MAP kinase could have a dual role in the cutaneous response to UVB. These data show that UVB, in addition to its deleterious effects on dermal matrix proteins, can also rapidly induce protective mechanisms in human epidermis directed at inhibition of serine proteinases and control of infection.

Introduction

Exposure to ultraviolet B light (UVB) has a strong impact on human skin, and causes acute and long term changes. Acute effects include altered gene expression, induction of inflammation and immunosuppression. Long term effects of chronic UVB exposure are carcinogenesis, and damage to dermal extracellular matrix proteins such as elastin and collagen. UVB exposure initiates a cascade of inflammatory events that modify gene expression profiles and alters the innate and adaptive immune system of the skin. The exact sequences of events and cellular interactions that are critical for induction of this response in vivo have not yet been fully elucidated. However, at the cellular level both the keratinocytes and the Langerhans cells, and at the molecular level DNA photoproducts and cytokines have been implicated as key players in this response. At the level of cellular signalling it was shown in vitro, using cultured keratinocytes, that cellular stress response pathways such as the MAP kinase cascade and the NFκB pathway are activated following UV irradiation [130,133,337-339]. Many studies have reported that a high dose of UVC radiation, to which human are not normally exposed, activates the JNK pathway [340]. More recent

studies have shown that also physiologically relevant UVB doses are able to activate p38, JNK and to a lesser extent ERK in cultured keratinocytes. The initial signal is probably generated at the cell surface and includes clustering of cell surface receptors for growth factors or cytokines [341] although the exact mechanism remains to be elucidated. Similarly, low doses of UVB were found to cause activation of NFκB, independently of chromosomal DNA damage [338,342]. Signalling by (pro)inflammatory cytokines has been suggested as a mechanism by which UVB effects are mediated [341,343-345]. Recently it was suggested that UVB induced signalling events such as activation of NFκB and AP-1 cause enhanced transcription of metalloproteinase genes, which leads to degradation of collagen and elastin [133,346,347]. Other examples of UVB induced alterations in gene expression pattern of human keratinocytes in vivo are the upregulation of ICAM-1 [348], upregulation of cytokeratins (CK)1/10 and 5/14 [349], and induction of CK16 [350]. The outcome of UVB irradiation of human skin is strongly dose-dependent. High doses of UVB will lead to the formation of apoptotic cells (sunburn cells) that is initiated via a p53 dependent pathway, and causes removal of severely damaged cells, thus minimising the risk of skin cancer [351,352]. Lower doses of UVB allow cell survival and repair of genetic damage, but the price of the cell survival response is a state of local and systemic immunosuppression, which in itself may be deleterious and contribute to cutaneous carcinogenesis [353,354].

In previous studies we have described the expression of genes in human epidermis that are involved in host protection during skin inflammation, barrier disruption and infection. We found that under such conditions (psoriasis, wound healing) the proteinase inhibitor SKALP/elafin, which protects against epidermolysis and was recently shown to have antibacterial properties [335], and SLPI a proteinase inhibitor with anti-microbial properties are strongly induced [294,310,356]. Recently we have found that induction of SKALP and SLPI gene expression in cultured keratinocytes is dependent on p38 MAP kinase activation (Pfundt *et al*, Arch.Dermatol.Res., accepted). Other studies have indicated that SKALP is transcriptionally regulated by AP-1 [301,330], whose activity is regulated by the c-jun N-terminal kinase (JNK) stress response pathway. Since SKALP/elafin and SLPI were found to be induced in human skin by osmotic and chemical stress, we were interested to know if these genes were also induced by UVB irradiation, and to determine the possible role of cellular stress response pathways herein. We found that UVB irradiation of human skin in vivo causes induction of SKALP and SLPI expression by the suprabasal keratinocytes, which is preceded by activation of cellular stress response pathways.

Material and methods

UV irradiation of human skin

A group of 9 healthy volunteers (18-36 years old) with no past or present history of skin diseases participated in this study. All subjects were irradiated using a Waldman UV 7001 k light cabin. For each individual the M.E.D. (minimal erythema dose (24 hrs. After irradiation)) was determined. The subjects were irradiated with three different doses of UV light, 0.5 M.E.D., 1 M.E.D. and 2 M.E.D.. Four-millimetre full thickness punch biopsies

were taken of the non-irradiated skin and 2 hrs, 4 hrs, 16 hrs, 24 hrs and 48 hrs after the skin had been irradiated. The biopsies were fixed in 3.8 % buffered formalin and embedded in paraffin. Six-μm sections of these biopsies were used for immunohistochemical characterisation.

Immunohistochemistry

Biopsies were fixed in buffered 3.8% formalin for at least 4 hours and processed for embedding in paraffin. Sections (6μm) were mounted on 3-aminopropyltriethoxy-silane (Sigma, St Louis MO, USA) coated slides. Sections were deparaffinized and rehydrated. For antigen retrieval from paraffin sections, the slides were pre-treated two times 5 min in 10mM citrate buffer for Ks8.12 (CK16), for MIB-1 (Ki-67) and for anti c-jun-p using a microwave oven (Miele, M720) at 450 Watt. For immunohistochemical detection of NFκB, SLPI/ALP and SKALP/elafin no pre-treatment was necessary. After preïncubation with 20% normal swine or rabbit serum the slides were incubated for 60 min with the primary antibodies and washed with phosphate-buffered saline (PBS). Samples for the detection of NFκB, phosphorylated c-jun, CK16 and Ki67 were subsequently incubated with the appropriate biotinylated secondary antibody for 30 minutes. To enhance the staining the sections were incubated with an Avidin-Biotin-Complex (ABC) after which they were developed with metal enhanced DAB as a chromogenic substrate. Samples for the detection of SLPI and SKALP were incubated with peroxidase conjugated secondary antibodies, and these sections were developed with aminoethylcarbazole (AEC) as chromogenic substrate. When desirable, the slides were counterstained with Mayer's haematoxylin (Sigma, St Louis, MO, U.S.A.) and mounted in glycerol gelatine. Apoptotic cells were identified using TUNEL staining as described before [357]. Immunohistochemical detection of phosphorylated p38 was performed using an enhancement kit for rabbit polyclonal antibodies (CSA kit, DAKO, CA, USA) as described by the manufacturer using polyclonal rabbit-anti-dually-phosphorylated-p38 (New England Biolabs, USA). Appropriate controls with pre-immune sera, blocking peptides or omission of the primary antibodies were performed.

Cell cultures

Primary keratinocytes were seeded in 6 well culture dishes and cultured until confluence in keratinocyte growth medium (KGM) as described before [77]. KGM was composed of keratinocyte basal medium (KBM (Biowhittaker, Walkersville, Maryland, USA); 0.15 mM Calcium), supplemented with ethanolamine (0.1 mM) (Sigma, St.Louis, USA), phosphoethanolamine (0.1 mM) (Sigma, St.Louis, USA), bovine pituitary extract (BPE; 0.4 $\%^{v}/_{v}$) (Biowhittaker, Walkersville, Maryland, USA), insulin (5 μg/ml), (Sigma, St. Louis, USA), hydrocortisone (0.5 μg/ml) (Collaborative Research Inc.), Recombinant Mouse Epidermal Growth Factor (EGF; 10 ng/ml) (Sigma, St. Louis, USA), penicillin (100 U/ml) (Gibco, Breda, the Netherlands) and streptomycin (100 μg/ml) (Gibco, Breda, the Netherlands). Induction of keratinocyte differentiation was established by switching the cells at confluence either to KGM supplemented with 5 $\%^{v}/_{v}$ FCS (KGM/FCS) (FCS from Seralab, Nistelrode, the Netherlands) resulting in regenerative differentiation or to KGM depleted of growth factors (BPE, Insulin, EGF and hydrocortisone (KGM-GF)) [265] resul-

ting in normal keratinocyte differentiation. Normally differentiated keratinocytes were stimulated either with Tumour Necrosis Factor-alpha (TNF-α (Boehringer Mannheim)), FCS (Seralab) or with UV-B irradiation. Ultra violet light was generated using a Fluo-Link (Vilber Lourmat) with a peak wavelength of 311 nm.

Enzyme-linked immunosorbent assay (ELISA) for measurement of SKALP/elafin levels

SKALP/elafin and ALP/SLPI concentrations in supernatants of cultured human keratinocytes were measured using sandwich-type ELISA. In short, microtiter plates (96 flat bottom wells) were coated overnight with either goat anti-SKALP/elafin antiserum or with monoclonal anti-SLPI (clone 31). After washing of the plate with PBS/0.05% Tween-20, microtiter plates were blocked and probed with the dilutions of test samples and standards for 2 hrs at 37˚C. As second antibodies we used either rabbit anti-SKALP/elafin antiserum or rabbit anti-SLPI antiserum with 2.5% normal goat serum (Vector Laboratories, Burlingame, USA). Subsequently the microtiterplates were incubated with peroxidase-conjugated swine-anti-rabbit IgG (DAKO, Glostrup, Danmark) with 2.5% normal goat serum during 60 minutes at 37˚C. Finally o-phenylenediamine dihydrochloride (OPD) (Pierce, Rockford, Illinois, USA) was added as chromogenic substrate for 15 minutes at RT. This enzyme reaction was stopped by the addition of 4 M H_2SO_4. Absorbencies were measured at 492 nm and at 655 nm. The SKALP/elafin and ALP/SLPI concentrations in the test samples were read from a calibration curve of recombinant SKALP/elafin or ALP/SLPI.

Western blotting

Using the phosphospecific antibodies kits supplied by New England Biolabs (Beverly, MA, USA) we performed Western blotting and protein detection according to the protocol of the manufacturer. In short, cultured keratinocytes were lysed in sample buffer ((approximately 5000 cells per µl) 62.5 mM Tris pH6.8, 10 % glycerol, 0.1 % bromphenol blue, 2 % SDS, 50 mM DTE). Cell lysates were sonified two times 10 sec. and subsequently incubated for 3 min. at 100˚C. Together with caleidoscopic and biotinylated markers, samples were run on a 10 % SDS PAGE gel (biorad) in Tris-Glycine-SDS buffer (Biorad) at 175 volts. Gels were electro-blotted on PVDF blotting paper (Biorad) in Tris-Glycine-SDS buffer supplemented with 20 % methanol at 400 mA for 35 min. After blocking using Blotto (Pierce, Rockford, Illinois, USA), the blots were incubated in the primary antibody solution (100 mM Tris pH7.5, 0.9 % NaCl, 5 % BSA, 0.1 % Tween-20, 0,1 % NaN_3) in which the phosphospecific antibody (either directed against c-jun phosphorylated at serine-73 site (New England Biolabs), against p38, dually phosphorylated both at the threonine-180 and the tyrosine-182 site (New England Biolabs) or against tyrosine phosphorylated ERK1/2 (New England Biolabs)) was diluted 1:1000. Blots were incubated in this solution for 2 hrs. at room temperature. After washing the blots in TBS (100 mM Tris, 0.9 % NaCl) the blots were incubated for 60 min. at room temperature in Blotto in which the secondary antibodies (against biotinylated marker and against primary antibody) were diluted 2000 times. Subsequently the blots were washed in TBS and incubated in chemiluminescent LumiGlo (New England Biolabs) reagent for 1 min. Finally the blots were

packed in food wrap and several exposures were made on Kodak X-Omat X-ray films. For quantification the Lumi-Imager system (Boehringer) was used.

Results

UVB irradiation of human skin causes induction of the regenerative epidermal differentiation program as witnessed by expression of SKALP, SLPI and CK16 whereas apoptosis is limited.
Normal human skin was irradiated with different doses of UV-B light (peak wavelength of 311 nm) ranging from 0.5 – 2 MED (1 MED is reached after irradiating an average skin type with 0.05-0.15 J/cm^2 of UV-B light). The effects of UV-B on the expression of host defence proteins SKALP/Elafin and ALP/SLPI, the differentiation specific proteins CK16 and involucrin and the proliferation rate of the epidermal keratinocytes were studied by immunohistochemistry. Twenty-four to 48 hours after irradiation we observed a high *de novo* expression of a number of proteins. SKALP, a protein that is otherwise absent from normal human epidermis (figure 1A) but is found in hyperproliferative skin (psoriasis, wound healing) is also expressed in the *stratum spinosum* after UV-B irradiation (figure 1B). SLPI, a proteinase inhibitor with antimicrobial properties, which is normally restricted to the granular layer (figure 1C), was also found in the stratum spinosum following UV-B irradiation (figure 1D). Involucrin, a structural protein involved in the formation of the cornified envelope, which is normally restricted to the granular layer (figure 1E), was also found in the stratum spinosum following UVB irradiation (figure 1F). In addition, in figure 1G/1H the induction of cytokeratin 16 a classical marker for epidermal activation is shown, indicative for a switch of differentiation program by these suprabasal keratinocytes. As can be seen in figure 1, the localisation of SKALP, SLPI and CK16 positive cells is distinct from that found in psoriatic epidermis where the entire stratum spinosum is positive. Following UV-B the upper layers remain negative and a band of positive cells is observed in the centre of the epidermis. As assessed by the expression of the proliferating nuclear antigen Ki67, within 24-48 hours the percentage proliferating keratinocytes in the basal layers of the epidermis increased from approximately 5% in normal skin (figure 1I) up to 80-90 % in skin irradiated with 2 MED of UV-B (figure 1J). This means that nearly all cells of the germinal layers are actively cycling, very similar to what is found in psoriatic skin or following skin injury.

Previous studies have shown that p53 mediated apoptosis is an important event in the surveillance against UV-B induced carcinogenesis [351,358]. At the doses used here (0.5 to 2 MED), a distinct increase in the number of p53 positive cells and in the intensity of p53 staining was found (not shown). The number of apoptotic cells as determined by counting of sunburn cells on H&E stained sections, or as determined by TUNEL staining was limited (figure 1K/1L). Only scattered apoptotic cells could be seen in the upper levels of the epidermis following 2 MED irradiation (figure 1L).

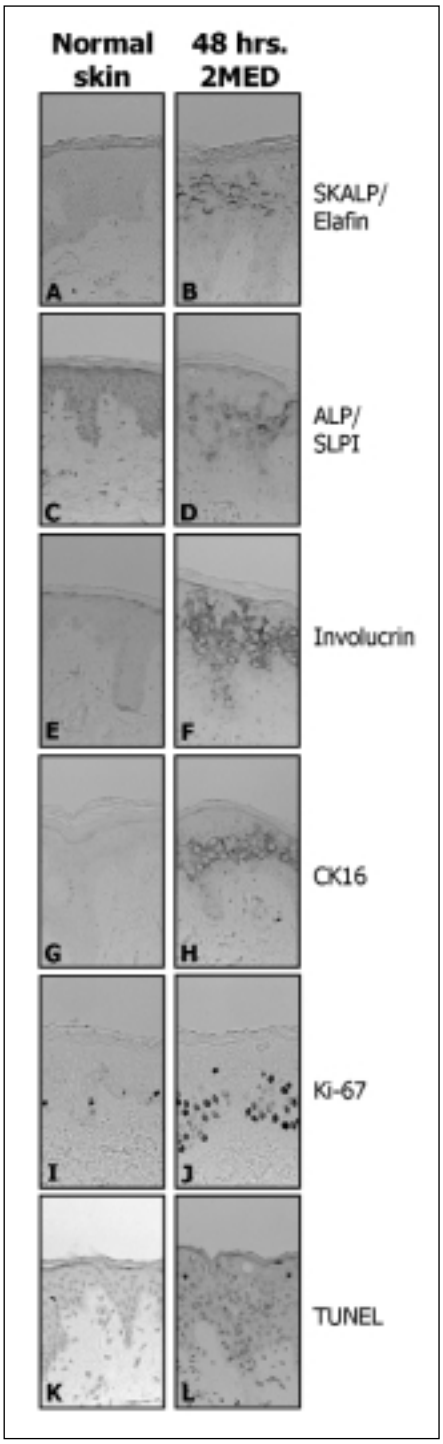

Figure 1. UV-B irradiation induces epidermal proliferation and expression of genes involved in host protection. Normal human epidermis does not express the proteinase inhibitor and antibacterial protein SKALP (1A). However 48 hours after 2 MED of UV-B irradiation high expression levels of SKALP can be detected in the suprabasal layers of the irradiated epidermis (1B). A similar effect can be seen for the proteinase inhibitor and antimicrobial protein SLPI (1C and 1D) although SLPI is also expressed in the granular layer of normal epidermis (1C). Involucrin, a protein involved in cornified envelope formation, is expressed in the *stratum granulosum* of normal epidermis (1E) whereas after UV-B irradiation it can be found throughout the suprabasal compartment (1F). The expression of cytokeratin 16, a marker for hyperproliverative epidermis in not expressed in normal skin (1G) but is also upregulated after UV-B irradiation (1H). Note that the expression of SKALP, SLPI, CK16 and to a lesser extent involucrin, is restricted to the middle layers of the stratum spinosum of the irradiated epidermis (1B/1D/1F/1H). In normal epidermis only 5 % of the basal keratinocytes is proliferative as assessed by the expression the nuclear antigen Ki67 (1I) 48 hours after irradiating the epidermis with 2 M.E.D. of UV-B light the complete basal layer stains positive for Ki67 indicating a massive recruitment of cells into the cell cycle (1J). Identification of apoptotic cells by TUNEL staining revealed no apoptotic cells in normal skin (1K) and few apoptotic cells in the upper layers of the stratum spinosum after mild (2 MED) UV-B irradiation (see arrows (1L)).

Stress response pathways are highly activated directly after UV-B irradiation
We used conventional and phospho-specific antibodies against several cell stress related proteins (p38, c-jun, and NFκB) in order to investigate the activity/localisation of these proteins in human epidermis in the course of the cellular response to UV-B irradiation. In normal skin phosphorylated c-jun can only be found in the granular layer of the epidermis where approximately 5 % of the cells stain positive (figure 2D). However when normal skin is irradiated with UV-B light (2 MED), within 2-4 hours massive levels of phosphorylated c-jun can be found throughout the whole epidermis (figure 2E). Even 24-48 hours after UV-B irradiation, levels of phosphorylated c-jun are still markedly elevated (figure 2F) indicating that c-jun phosphorylation is an immediate response to UV-B that persists for a longer period of time. Phosphorylated p38 MAP kinase can not be detected in the keratinocytes of normal skin (figure 2A). When the skin is irradiated with UV-B light (2 MED) within 2 hours high levels of dually phosphorylated p38 can be detected (figure 2B). Unlike c-jun these phosphorylation levels return back to almost normal within 16-24 hours (figure 2C) suggesting that p38 activity is a rather early response to UV-B irradiation *in vivo*. In normal skin the transcription factor NFκB is mainly localised in the cytosol where it is held inactive by binding to IκB (figure 2G). When skin is irradiated with UV-B light (2 MED) there is a translocation of NFκB to the nuclei of the keratinocytes as observed by profound nuclear staining of the keratinocytes 2 hours after the irradiation (figure 2H). This nuclear staining decreases already after 4 hours (not shown) and returns to normal levels within 16-24 hours after skin irradiation (figure 2I). Taken together, these results indicate that a number of proteins that are part of the mammalian stress response pathway are activated prior to the transition from normal to regenerative epidermal differentiation.

UV-B irradiation of cultured keratinocytes is not sufficient to induce SKALP or SLPI expression in vitro
In order to further study the role of UVB on the activation of epidermal keratinocytes we used a culture model that we have described previously which allows normal differentiation in a submerged system [77]. Confluent keratinocyte cultures were allowed to differentiate for 48 hours by depleting the culture medium of its growth factors. These differentiated keratinocyte cultures were then irradiated with a non-cytotoxic doses of UV-B light (0.015-0.025 J/cm^2; peak wavelength 312 nm). As a positive control we used TNF-α (25 ng/ml) or 5% FCS which were previously shown to be potent inducers of SKALP [294] and SLPI [356] gene expression. In contrast to TNF-α and FCS, UV-B irradiation did not induce significant increases of SKALP or SLPI protein in the culture media, up to 48 h (figure 3). When higher doses of UV-B were applied this resulted in cytotoxicity as determined by LDH release (not shown).

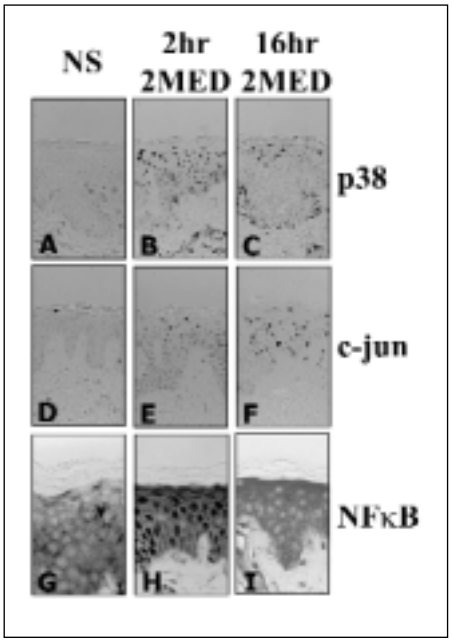

Figure 2. Members of stress response pathways are activated upon UV-B irradiation of human skin in vivo. In normal skin stress response proteins like p38 or c-jun are not present in their phosphorylated (activated) forms (2A and 2D). However when skin is irradiated with UV-B light (2MED) within 2 hours phosphorylation of these proteins is already detected (2B and 2E). p38 is dephosphorylated within 16 hours after irradiation (2C) whereas high levels of phosphorylated c-jun remain in the epidermis until 16-24 hours after UV-B irradiation (2F). In addition to phosphorylation of c-jun and p38 also the cellular stress associated transcription factor NFκB is being activated and translocated to the nucleus. In normal skin it is mainly localized in the cytoplasm of the keratinocytes (2G). Whereas upon UV-B irradiation within 2 hours translocation of NFκB to nuclei was found (2H). Sixteen hours after the irradiation NFκB is localized again mainly is the cytoplasm (2I).

UV-B irradiation of keratinocytes results in a rapid and extensive phosphorylation of p38 MAP kinase and c-jun

The in vitro findings suggested that UV-B induced expression of SKALP and SLPI in vivo, as described above, was dependent on additional factors that were absent in culture. Because from previous studies (Pfundt et al, submitted) it was known that SKALP expression, induced by FCS or TNF-α, was dependent on p38 MAP kinase activity and studies on the SKALP/elafin promoter have suggested a role for AP-1 [301,330], we investigated whether activated p38 MAP kinase and c-jun could be found in the UV-B irradiated cultures. Using western blot analysis with the phosphorylation specific antibodies against p38 and c-jun we checked the activation status of these proteins in cultured keratinocytes at different time points after UV-B irradiation. Like normal skin *in vivo*, in normal differentiated cultured keratinocytes the basal levels of phosphorylated c-jun are low (figure 4, panel C, lane 1). However upon UV-B irradiation, within 30 minutes, the phosphorylation levels of c-jun strongly rise (figure 4, panel C, lane 2). This increase persists for 48 hours after irradiation (figure 4, panel C, lanes 3-5) and drops close to initial levels 72 hours after UV-B irradiation (figure 4, panel C, lane 6), more or less similar to the *in vivo* situation. In normal differentiated cultured keratinocytes detectable levels of phosphorylated p38 are present (figure 4, panel A, lane 1). Like c-jun, p38 is heavily phosphorylated shortly after the keratinocytes have been irradiated with UV-B (figure 4, panel A, lane 2). This increase in phosphorylation status of p38 lasts for at least 6 hours after irradiation (figure 4, panel A, lanes 3-4). Normal p38 phosphorylation levels are reached 48 hours after irradiation, indication that, as *in vivo*, activation of p38 is an early response to UV-B

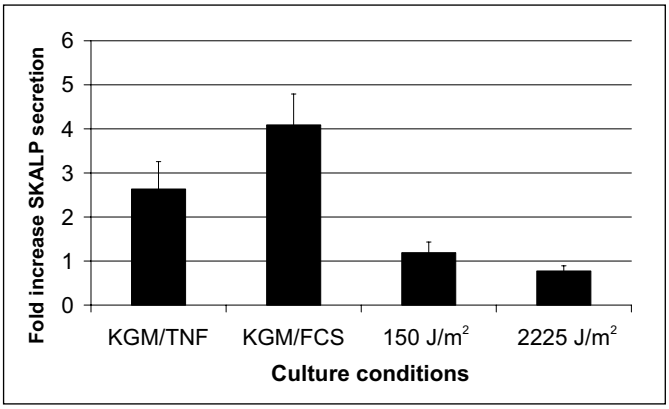

Figure 3. UV-B irradiation does not directly induce SKALP or ALP gene expression in cultured human epidermal keratinocytes. The induction of SKALP secretion was measured 48 hours after stimulating the cells with TNF-α, FCS or UV-B irradiation (150 and 225 J/m^2). The concentration SKALP secreted in the culture medium by the keratinocytes was determined using a sandwich-type ELISA. The concentration of SKALP secreted in the medium is given as a fold-increase relative to unstimulated cells. Treatment of keratinocytes with FCS or TNF-α results in SKALP secretion whereas SKALP is not induced by UV-B irradiation. Error bars indicate the standard error of the mean (SEM).

irradiation. The kinetics of p38 and c-jun phosphorylation are distinct from those found following the addition of FCS or TNF-α, which do not cause an immediate burst of phosphorylation, but only a mild, gradual increase (Pfundt *et al*, Arch.Dermatol.Res., accepted).

Inhibition of p38 directly after UV-B irradiation *in vitro* results in massive cell death
From the experiments described in the previous section it was clear that the lack of SKALP induction by UV-B in vitro was not due to an absence of p38 activation, since huge levels of phosphorylated p38 were found directly after UV-B irradiation. We therefore considered the possibility that UVB-induced p38 hyperphosphorylation could still be involved in regulation of basal levels of SKALP expression, or alternatively could be a negatively regulating factor. Surprisingly, treatment of keratinocytes with a pharmacological p38 inhibitor (SB202190) directly after or before UV-B irradiation results in massive cell death. Cell death was obvious by morphological criteria, and was quantified by measurement of LDH release in the media (see figure 5). The same concentrations of these inhibitors are non-cytotoxic for keratinocytes cultured under basal conditions, or when stimulated with FCS or TNF-α. When keratinocytes are treated with SB202190, 24 hours after exposure to UV-B, significantly lower levels of cytotoxicity were observed (not shown). These observations suggest that the early activation of p38 in response to UV-B irradiation could be important in processes involved in cell survival after UV-B irradiation.

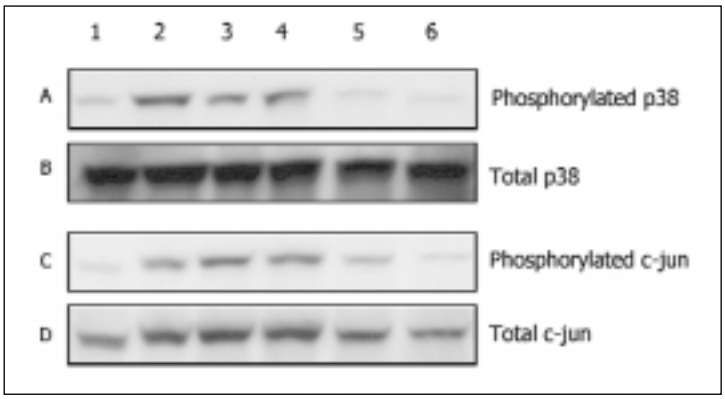

Figure 4. UV-B irradiation of cultured human epidermal keratinocytes results in the activation of stress response pathways. The phosphorylation status on c-jun and p38 was assessed by western blot analysis using phosphorylation specific antibodies. In normally differentiated keratinocytes in vitro, low levels of phosphorylated (activated) p38 and c-jun can be found (lanes 1 panels A and C). Upon UV-B irradiation (150 J/m^2) p38 and c-jun are rapidly (within 30 min) phosphorylated (lanes 2). The levels of phosphorylated c-jun and p38 remain equally high 60 min (lanes 3) and 240 min (lanes 4) after UV-B irradiation. After 48 hours (lanes 5) the phosphorylation levels of p38 returned back to the initial level (panel A) whereas the level of phosphorylated c-jun remains high (panel C). After 72 hours also the level of phosphorylated c-jun returns back to starting level (lane 6, panel C). As a reference for total amounts of c-jun and p38 in the keratinocytes (phosphorylated as well as unphosphorylated) western blot analysis was also performed using antibodies recognizing both phosphorylated and non-phosphorylated forms of the proteins (panels B and D).

Discussion

In a number of *in vivo* and *in vitro* studies, UVB-induced activation of cellular stress response pathways such as the MAP kinase cascade and the NFκB route has been demonstrated in cultured keratinocytes or in vivo in whole skin extracts using biochemical assays. The activation of these proteins has been implicated in several physiological responses to UVB irradiation. JNK activation has been implicated in the expression of several AP-1 dependent genes like e.g. the matrix metalloproteases. It has been demonstrated that repression of AP-1 formation by retinoic acid prevents the expression of these proteinases. These findings, that offer an explanation at the molecular level for UVB induced photodamage to dermal collagen and elastin [133,134], prompted us to investigate whether the opposite was also true, i.e. the induction of protective mechanisms by UVB. Our studies show that SKALP and SLPI, which are both implicated in host defence and protection [294,356], are highly upregulated. In addition, this is, to our knowledge, the first *in situ* demonstration of the cellular source of the phosphorylating activities induced upon UVB irradiation. Using phosphorylation-specific antisera we found that high levels of phosphorylated p38 MAP kinase and c-jun can be found in the nuclei of basal and suprabasal keratinocytes. Previous studies by others and us have shown that p38 and c-jun (being a component of the AP-1 complex) are involved in transcriptional regulation of

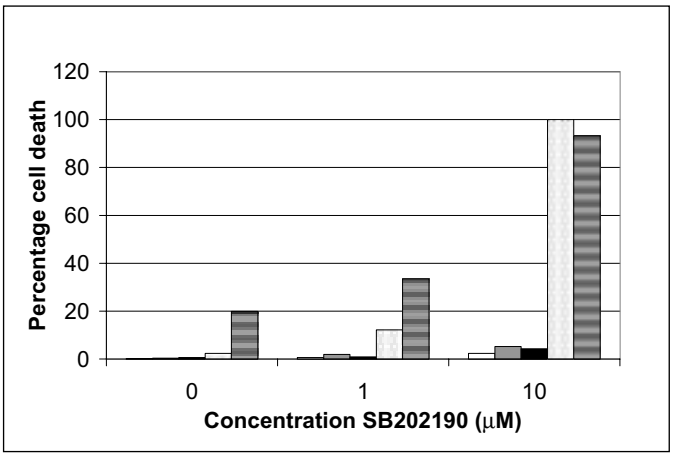

Figure 5. Inhibition of p38 activity leads to massive cell death in response to UV-B irradiation. The cytotoxic effect of the inhibition of p38 directly after stimulating the cells with FCS, TNF-α or UV-B irradiation was measured. As a read out for cytotoxicity, LDH activity was measured in the culture medium 48 hours after stimulation. When unstimulated cells (black bars) or cell stimulated with FCS (white bars) or TNF-α (gray bars) were treated with a p38 inhibitor, low levels of cytotoxicity could be measured after 48 hours (<5% at 10 μM SB202190). However when cells that were irradiated with UV-B (150 J/M^2 (dotted bars) or 225 J/m^2 (striped bars)) were treated with SB202190, high levels of cytotoxicity could be found after 48 hours (up to 100% at 10 μM SB202190). Note that irradiation with 225 J/m^2 results in 20 % cytotoxicity even when cells are not treated with SB202190.

SKALP [330,301] (Pfundt et al, submitted). Moreover, AP-1 has been implicated in the regulation of a significant number of epidermal genes [326]. Our observations that c-jun phosphorylation levels rise quickly after UV-B irradiation and remain high for several days indicate that c-jun complexes could be involved both in early immediate and late adaptation processes. This in contrast to p38 activation, which is only an early event in response to UV irradiation, already 48 hours after UV-B irradiation, the levels of phosphorylated p38 drop to initial amounts. In our in vitro studies we could not find a direct effect of UVB irradiation on cultured keratinocytes, with respect to SKALP expression. This suggests that in vivo other secondary factors generated by UVB are required. Two major inducers of SKALP expression in vitro (serum and TNF-α) are known to be present in inflamed skin. Firstly, Andriessen *et al* [359] have shown that during inflammation, plasma proteins from the oedematous dermis enter the epidermal compartment which is normally impermeable for high molecular weight proteins from the circulation. Secondly, Strickland *et al* [360] have shown that UVB irradiation of human skin causes a strong upregulation of TNF-α. We would hypothesise that TNF-α production and oedema-formation resulting from UVB irradiation can induce SKALP and SLPI expression. Our in vitro experiments revealed a second, rather unexpected finding. Inhibition of p38 activity by SB202190 at non-cytotoxic concentrations inhibits serum or TNF-α induced SKALP expression, as we have shown before. However when SB202190 was applied shortly before

or after UVB irradiation massive cell death was observed as assessed by LDH release. Because only a minor (two-fold) increase in the number of TUNEL positive cells was observed, and a mild increase in caspase activity (not shown) most of the cells appear to die without going into apoptosis. The exact mechanism and kinetics of UVB induced cell death in combination with p38 inhibition require further investigation. These observations are in contrast with recent data on a pro-apoptotic effect of p38 [141].

An important conclusion that can be drawn from our observations is that instead of being merely subject to local and systemic responses to UVB, keratinocytes are very active players in this process. It becomes more and more clear that keratinocytes can express an impressive array of proteins with various protective properties. Either constitutively, or upon cellular stress the epidermal keratinocytes can load the epidermis and dermis with antiviral, antibacterial and anti-inflammatory activity, thereby regulating immune responses and fighting potential pathogens [355,356,361-364]. SLPI, originally described as an inhibitor of leukocytic proteinases also displays antiviral and antibacterial activity and a recent study has identified SLPI as an anti-inflammatory protein that interferes with NFκB activation [365]. We have previously shown that SLPI associates with the dermal elastic fibers, and preliminary investigations have shown that upon chronic UVB irradiation, SLPI accumulates in the dermis, thereby protecting against elastolysis (Pfundt et al, unpublished observations). Similarly we have shown in a previous study that SKALP expression by keratinocytes can directly result in protection against elastase mediated tissue destruction [294]. I a recent article of Zaidi et al overexpression of SKALP in a transgenic mouse results in protection against elastase mediated cardiac dysfunction [366]. All these studies show that proteinase inhibitors like SKALP and SLPI play crucial roles in regulating acute inflammatory responses. Although chronic exposure of human skin to UVB will eventually lead to photodamage of dermal matrix proteins, UVB can apparently also recruit cellular defence mechanisms. Our data illustrate that UVB-induced metalloproteinase induction and serine proteinase inhibitor induction are two sides of the same coin.

Tenascin-C expression in human epidermal keratinocytes is regulated by inflammatory cytokines and a stress response pathway.

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Abstract

Recently we showed that human epidermal keratinocytes express the extracellular matrix protein tenascin-C during wound healing, but not in normal adult skin. In order to gain further insight into the regulation of epidermal tenascin-C expression, we tested the effect of various stimuli on tenascin-C expression by cultured keratinocytes. Our results indicate that IL-4 is a very strong inducer of tenascin-C protein and mRNA expression in normal keratinocytes. Furthermore, TNF-α and IFN-γ moderately increased tenascin-C expression. All other cytokines and growth factors that we tested, including various factors that stimulate tenascin-C expression in mesenchymal cells, did not significantly affect tenascin-C secretion by cultured keratinocytes. The regulation of tenascin-C expression in keratinocytes is distinct from that of fibronectin, since IL-4 and IFN-γ did not affect fibronectin expression in our experiments and TNF-α only slightly increased fibronectin levels. To investigate the role of cellular stress response pathways that can be activated by TNF-α in the regulation of tenascin-C expression, we tested the effect of different inhibitors and an activator of these intracellular signalling cascades. The results show that the p38 MAP-kinase pathway is not involved in TNF-α-induced tenascin-C expression in cultured keratinocytes. Activation of the JNK/SAPK-1 pathway by the addition of sphingomyelinase resulted in a dose-dependent increase of tenascin-C expression. Tenascin-C expression by squamous carcinoma cell lines was differentially affected by the cytokines that stimulated tenascin-C expression in normal keratinocytes: TNF-α again increased tenascin-C secretion, but IL-4 and IFN-γ had little effect. We conclude that there are distinct regulation mechanisms for tenascin-C expression in normal keratinocytes, tumour-derived keratinocytes and mesenchymal cells. The observation that tenascin-C is abundant in inflamed skin is a strong indication that inflammatory cytokines such as IL-4, TNF-α and IFN-γ could also be involved in the regulation of epidermal tenascin-C expression in vivo.

Introduction

Tenascin-C is a large hexameric extracellular matrix glycoprotein with a modular structure. For review see [367,368]. Tenascin-C is abundant during embryogenesis, particularly at epithelial-mesenchymal interaction sites, but expression is limited in adult healthy tissues. In adult human skin, tenascin-C is present in the dermis where it is sparsely distributed at the dermal-epidermal junction and surrounds blood vessels and epidermal adnexal structures [369-371]. Tenascin-C expression in skin is markedly increased, however, in pathologic conditions that are characterised by inflammation and epidermal hyperproliferation, like psoriasis [372], epidermal tumours [373,374], UV-irradiation [375,376] and injury [370,377,378,] (and references herein). In vitro studies suggested various functions for tenascin-C, including modulation of cell proliferation, differentiation, adhesion and migration, and suppression of the immune response [367,368]. The role of tenascin-C in vivo, however, remains elusive especially because knocking out the tenascin-C gene in mice caused no marked phenotypic changes [379,380]. Interestingly, however, in man deficiency for tenascin-X, which is anot-

her member of the tenascin family, affects skin, is associated with Ehlers-Danlos syndrome [381].

Previously we showed that tenascin-C expression in human skin is not confined to mesenchymal cells in the dermis: epidermal keratinocytes can produce tenascin-C as well, and they do so in vivo during wound healing and when cultured in vitro, but not in normal non-wounded skin [377]. Aukhil and colleagues also observed epidermal tenascin-C expression in healing wounds in rat [382]. In other studies, tenascin-C protein [383-385] and mRNA [386] was detected in epidermal tumour cells. These data indicate that keratinocytes can also produce tenascin-C in vivo in case of malignancy.

Many factors have been described that modulate tenascin-C expression, and the regulation of tenascin-C expression seems to be cell-type specific (reviewed in [367,387]). Among the factors that stimulate tenascin-C expression by mesenchymal cells and tumour cell lines are TGFβ1, EGF, KGF, PDGF, bFGF, TNF-α, IL-1α, and IL-4. Knowledge of the regulation of tenascin-C expression by skin cells, however, is limited. Immunohistochemical studies showed that tenascin-C in skin is more abundant in conditions that are characterised by inflammation and epidermal hyperproliferation, suggesting that inflammatory cytokines and growth factors could be involved in the induction of tenascin-C expression. Recently Makhluf et al. indeed showed that addition of IL-4, and to a lesser extent PDGF and bFGF, increased tenascin-C expression by cultured skin fibroblasts [388], but no data are available yet with respect to keratinocytes. In order to gain further insight into the regulation of tenascin-C expression by epidermal keratinocytes, we tested the effect of growth factors and cytokines on tenascin-C expression in an in vitro model using cultured keratinocytes. The results show that tenascin-C expression by keratinocytes is upregulated by various inflammatory cytokines. Regulation of expression in normal keratinocytes seems to be distinct from the regulation in tumour-derived keratinocytes and skin fibroblasts.

Materials and methods

Cells and culture conditions

Human epidermal keratinocytes from skin biopsies of healthy donors were initially cultured according to the method of Rheinwald and Green as previously described [224,377]. In this study we used second passage normal keratinocytes that were grown in serum-free keratinocyte growth medium (KGM). KGM was composed of KBM (keratinocyte basal medium, Clonetics, San Diego, CA; 0.15 mM Calcium) supplemented with 0.1 mM ethanolamine (Sigma, St.Louis, MO), 0.1 M phosphoethanolamine (Sigma), 10 ng/ml EGF (recombinant mouse EGF; Sigma), 5 μg/ml insulin (Sigma), 0.5 μg/ml hydrocortisone (Collaborative Research Inc., Lexington, MA), 0.4% $^{v}/_{v}$ bovine pituitary extract (Clonetics), and 100 units/ml penicillin plus 100 μg/ml streptomycin (Gibco, Breda, the Netherlands). To investigate the effect of human cytokines and growth factors on tenascin-C secretion, keratinocytes were seeded in 6-well plates in KGM and grown until the cultures were nearly confluent. Then, the medium was changed for KGM/-GF to which the factor to be tested was added. KGM/-GF is KGM without growth factors or hydrocortiso-

Table 1

Compound	Concentration range tested
FCS	5%
IL-1α	0.04 - 10 ng/ml
IL-2	0.03 - 17 units/ml
IL-4	1.95 - 1000 units/ml
IL-6	0.78 - 400 units/ml
IL-8	0.97 - 500 ng/ml
IL-10	0.36 - 22.8 ng/ml
TNF-α	0.16 - 83 ng/ml
IFN-γ	15.6 - 1000 units/ml
TGF-α	0.02 - 10 ng/ml
TGF-β	0.04 - 20 ng/ml
EGF	0.02 - 10 ng/ml
KGF	0.20 - 100 ng/ml
PDGF	0.10 - 50 ng/ml
bFGF	0.31 - 20 ng/ml
SMase[1]	1.50 - 200 munits/ml
SB203580[2]	0.63 - 10 μM
SB202190[2]	0.63 - 10 μM
PD98059[3]	1.60 - 25 μM

1 SMase stands for sphingomyelinase
2 SB203580 and SB202190 specifically inhibit p38 MAP kinase
3 PD98059 specifically inhibit the MAP kinase kinases MEK1 and MEK2, which activate the ERK MAP kinases

ne (i.e. KBM supplemented with 0.1 mM ethanolamine, 0.1 M phosphoethanolamine, 100 units/ml penicillin and 100 μg/ml streptomycin). Medium was collected after 48 hours and tenascin-C concentrations were determined by means of an enzyme-linked immunosorbent assay (ELISA). The cells of the cultures were washed with PBS and processed for RNA-isolation. The human cytokines and growth factors that were tested are summarised in Table I. IL-1α and IL-10 were kindly provided by L. Joosten (Rheumatology Department, University Hospital, Nijmegen, the Netherlands). IL-4 was a kind gift from Schering & Plough (Amstelveen, The Netherlands; accepted for research purposes only), and IL-6 was a kind gift from Dr. L. Aarden (Central Laboratory for Bloodtransfusion, Amsterdam, the Netherlands). IL-8, TGF-α, TGF-β, and IFN-γ were obtained from R&D systems (Abingdon, U.K.) while IL-2, bFGF, PDGF, KGF, and TNF-α were from Boehringer (Mannheim, Germany). Recombinant human EGF was kindly provided by Dr. E. van Zoelen (Cell Biology Department, University of Nijmegen, the Netherlands). In addition, we tested the effect of foetal calf serum (FCS; Seralab, Nistelrode, the Netherlands) on tenascin-C expression.

To compare the regulation of tenascin-C expression between normal keratinocytes and transformed keratinocytes derived from epithelial tumours, we investigated tenascin-C secretion by the squamous cell carcinoma cell lines SCC-4, SCC-12 and SCC-15 that were a kind gift of Dr. J. Rheinwald (Division of Dermatology, Harvard Medical School, Boston,

MA). SCC-4 and SCC-15 are derived from squamous cell carcinomas of the tongue, while SCC-12 is derived from a cutaneous squamous cell carcinoma [389]. Cell lines were initially cultured on a feeder layer of irradiated 3T3 cells in DMEM/F12 (3:1, $^{v}/_{v}$; Flow Laboratories, Irvine, Scotland, U.K.) supplemented with 0.4 (g/ml hydrocortisone, 5% FCS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Keratinocytes from these cultures were subsequently seeded in 6-well plates in the same medium but without feeder. After 3-6 days, the medium was changed for KGM in which the cultures were grown until near confluence. Then, the cells were incubated with KGM/-GF to which the factor to be tested was added. After 48 hours, medium was collected to determine tenascin-C concentrations by ELISA, and cells were harvested for RNA-isolation.

Kinase agonists and antagonists

To investigate intracellular signalling events involved in the stimulation of tenascin-C expression by TNF-α, we tested the effects of specific inhibitors of intracellular phosphorylation cascades involved in the regulation of gene expression by TNF-α and other stimuli [307]. SB203580 and SB202190 specifically inhibit the mitogen-activated protein (MAP) kinase p38, while PD98059 inhibits the MAP kinase kinases MEK1 and MEK2 (where MEK stands for mitogen-activated, extracellular-signal-related kinase) that can phosphorylate the MAP kinases p42 and p44 (also known as ERK1 and ERK2, which stands for extracellular-signal-related kinases 1 and 2). The third of the three MAP kinase pathways known to date involves the MAP kinase JNK (c-jun amino-terminal kinase), also referred to as SAPK-1 (stress-activated protein kinase 1). For this MAP kinase pathway, no specific inhibitor was available. Instead, we tested whether activation of the JNK/SAPK-1 pathway by the addition of sphingomyelinase affected tenascin-C expression. The inhibitors SB203580, SB202190 or PD98059 were added to confluent cultures of normal keratinocytes in KGM/-GF, one hour prior to the subsequent addition of TNF-α or sphingomyelinase. Medium was harvested after 48 hours and tenascin-C levels were measured by ELISA. To check for possible cytotoxic effects of the inhibitors, lactate dehydrogenase levels in the medium were determined using the Cytotoxicity Detection Kit (LDH) from Boehringer (Mannheim, Germany) according to the manufacturer's instructions. SB203580 was a kind gift of Dr. D. Griswold (SmithKline Beecham Pharmaceuticals, King of Prussia, PA). SB202190 was obtained from Calbiochem (La Jolla, CA) and PD98059 from Biomol (Plymouth Meeting, PA). The sphingomyelinase preparation that we used was purified from *Staphylococcus aureus* and obtained from Sigma-Aldrich Chemie (Zwijndrecht, the Netherlands). The test concentrations of the compounds are mentioned in Table I.

ELISA

To quantify tenascin-C concentrations in conditioned medium, a modification of the sandwich type ELISA that we developed previously was applied . Normal goat serum (1% in PBS) was used instead of BSA to block free protein binding sites and binding of the secondary antiserum against tenascin-C was assessed with the peroxidase Vectastain Elite ABC-kit (Vector Laboratories, Burlingame, CA) to make the assay more sensitive. The detection limit was approximately 5 ng tenascin-C per ml.

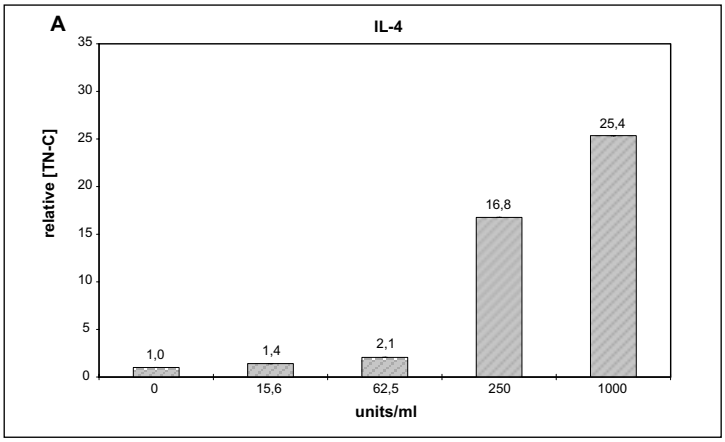
Fibronectin concentrations were measured according to the sandwich-ELISA method that we described previously [377]. The detection limit was approximately 80 ng fibronectin per ml.

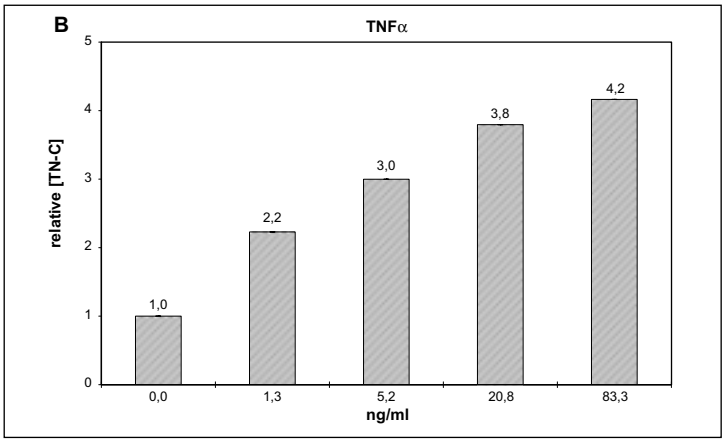
Construction of plasmid and DNA probe synthesis
Total RNA was isolated from cultured human dermal fibroblasts and cDNA was synthesised by reverse transcription with oligo-dT primers. By means of a polymerase chain reaction part of the cDNA was amplified. The PCR-product, corresponding to nucleotides 6015-6647 of the human tenascin cDNA-sequence as submitted by Gherzi and colleagues to the EMBL Database Library (accession number X78565) and thus spanning part of the most C-terminally located fibronectin type III repeat and part of the fibrinogen-like domain, was cloned in pGEM-3. The insert was used to synthesise ^{32}P-dCTP labelled tenascin-C probes with Klenow DNA-polymerase and random hexanucleotides.

RNA isolation and Northern blot analysis
Total RNA was isolated from cultured cells according to the guanidium-thiocyanate method as previously described (Pfundt et al., submitted). As a positive control we used total RNA from the human glioblastoma cell line U138-MG that is known to express tenascin-C at high levels [377,390]. 15 μg total RNA was dissolved in 10 mM sodium phosphate (pH 7.0), 1 M glyoxal, 50% DMSO, and electrophoretically separated in a 1% agarose gel in 10 mM sodium phosphate buffer (pH 7.0) [391]. The gel was blotted by capillary transfer onto a positively charged nylon membrane (Boehringer, Mannheim, Germany) using 10x salt sodium citrate (10x SSC consists of 1.5 M NaCl and 0.15 M trisodium citrate). After transfer, the blot was rinsed in 2x SSC, and RNA was fixed to the membrane by ultraviolet irradiation (312 nm, 0.2 J/cm^2). To visualise the molecular weight markers (5 μg of glyoxalated HindIII-digested λ-DNA [391] and ribosomal RNA bands, the blot was stained with 0.03% (w/v) methylene blue in 0.3 M sodium acetate (pH 5.2). After photography the blot was destained in water, and glyoxal-guanosine adducts were dissociated by incubation with 20 mM Tris (pH 8.0) for 5 minutes at 65˚C. Pre-hybridisation and hybridisation were performed at 65˚C. Control hybridisation for equal RNA loading was performed using a 28S human ribosomal RNA probe. For processing of the autoradiographs the Imagemaster™ data image system (Pharmacia, Biotech Inc., Uppsala, Sweden) was used.

Results

Inflammatory cytokines increase tenascin-C expression of keratinocytes
Previously we reported that tenascin-C mRNA could not be detected by in situ hybridisation in the epidermis of normal skin, while epidermal keratinocytes do express tenascin-C during wound healing and also in vitro [377]. We therefore were interested to investigate which stimuli would regulate the expression of tenascin-C by keratinocytes in vitro. To rule out major effects of the stimuli on keratinocyte proliferation and consequently on cell number, cultures were grown till confluence before addition of the agents. After 48 hours, the conditioned medium was harvested and the tenascin-C concentration was determined





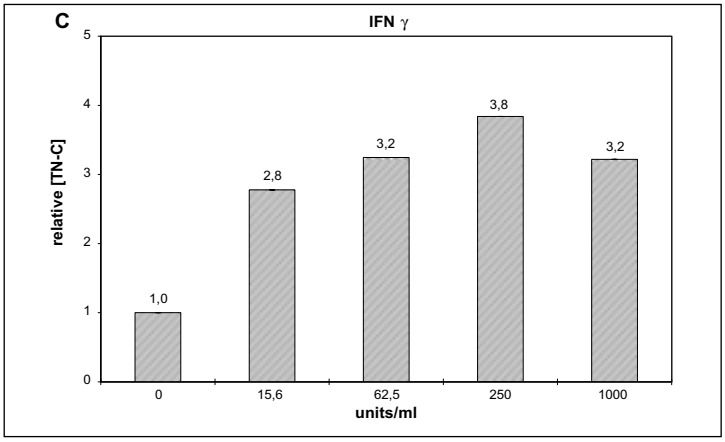


Figure 1. IL-4, TNF-α and IFN-γ increase tenascin-C secretion by cultured normal keratinocytes. Confluent cultures were incubated with IL-4 (A), TNF-α (B) or IFN-γ (C) in medium devoid of any other growth factors or cytokines. After 48 hours medium was harvested and tenascin-C concentrations were determined by ELISA. Values were normalised, and expressed relative to the tenascin-C concentration in the medium of control cultures without cytokine which was 48 ± 3 ng/ml. Data shown represent mean values of triplicate experiments ± SD.

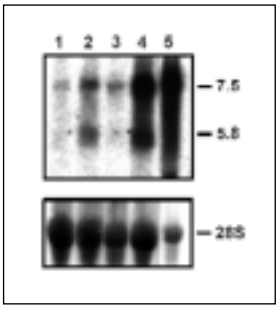


Figure 2. TNF-α, IFN-γ and IL-4 increase tenascin-C mRNA. Total RNA (15 μg) from normal keratinocytes cultures was incubated with a ^{32}P-labeled cDNA probe specific for human tenascin-C. As a control, 15 μg total RNA from the human glioblastoma cell line U138-MG, which is known to express tenascin-C at high levels, was used. In addition, hybridisation with a ^{32}P-dCTP labeled human 28S rRNA probe is shown as a control for RNA loading. Lane 1; keratinocytes cultured in KGM/-GF without cytokines. Lanes 2-4; keratinocytes cultured in KGM/-GF in the presence of 83.3 ng TNF-α per ml, 1000 units IFN-γ per ml, and 1000 units IL4 per ml, respectively. Lane 5; U138-MG cells.

by ELISA. Addition of 5% FCS to basal medium (KGM or KGM/-GF), induced a minor increase in tenascin-C levels. TGFα, TGFβ1, EGF, KGF, PDGF and bFGF, which increase tenascin-C expression in mesenchymal cells and tumour cell lines, did not significantly affect tenascin-C secretion by the cultured keratinocytes, not even at high concentrations (see Table I). Also IL-1α, IL-6, IL-8 and IL-10 did not significantly alter tenascin-C levels in the medium. In contrast, TNF-α and IFN-γ moderately increased tenascin-C concentrations in the medium, and addition of IL-4 resulted in a marked increase in tenascin-C levels (figure 1).

To investigate the effect of selected cytokines on tenascin-C mRNA levels, total RNA was isolated from keratinocyte cultures that were also used to quantify tenascin-C protein levels in the culture supernatant. Tenascin-C mRNA was analysed on northern blots that were hybridised with a tenascin-C specific probe (see figure 2). In control RNA samples derived from a human glioblastoma cell line that expresses tenascin-C at high levels, a major RNA band of approximately 7500 nucleotides hybridised with the tenascin-C probe. A second band of approximately 5800 nucleotides was only visible after prolonged exposure. Because of the long exposure times needed to visualise tenascin-C mRNA-bands in keratinocyte samples, the two individual tenascin-C bands in the glioblastoma RNA can not be discerned properly in figure 2. In RNA samples from control cultures of normal keratinocytes without cytokines, two tenascin-C mRNA bands of approximately 7500 and 5800 nucleotides were detected. In samples from keratinocyte cultures that were incubated with TNF-α, IFN-γ or IL-4, both tenascin-C mRNAs were markedly upregulated. The amount of tenascin-C RNA was quantified and normalised for the amount of RNA loaded, by measuring the optical density of the hybridisation signals on the autoradiograph. TNF-α increased the amount of tenascin-C mRNA approximately 5 times compared to the control culture without cytokine. IFN-γ upregulated tenascin-C mRNA approximately 3 times, and in the presence of IL-4, about 9 times as much tenascin-C mRNA was expressed. Note that for IL-4, the increase in tenascin-C mRNA is considerably smaller than the increase in tenascin-C protein that we measured in the medium of that culture. This could be explained by the fact that the mRNA is examined at a single time, whereas the protein detected by ELISA reflects tenascin-C that is synthesised and secreted into the medium during 48 hours. For TNF-α- and IFN-γ-stimulated cultures, increase in tenascin-C mRNA is nearly similar to the rise in tenascin-C protein levels.

Co-stimulation of normal keratinocytes with submaximal concentrations of TNF-α

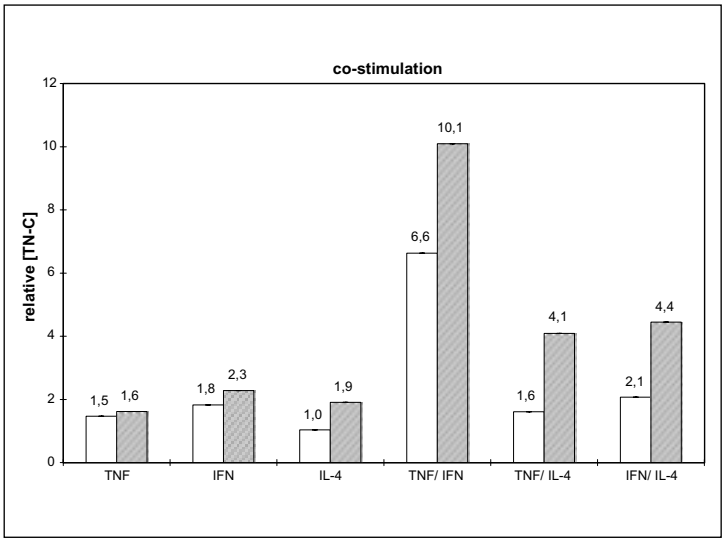
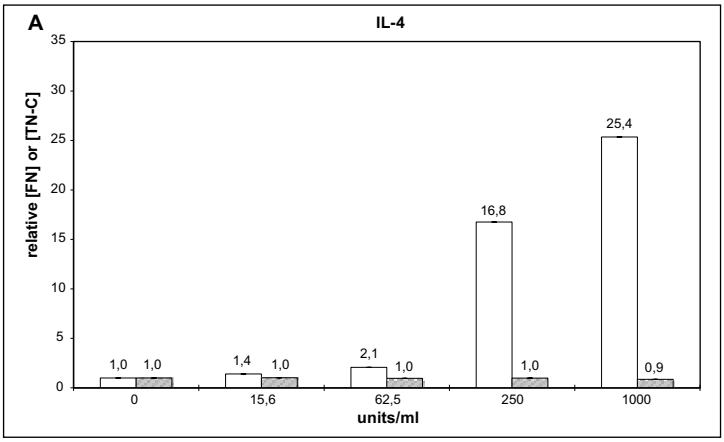


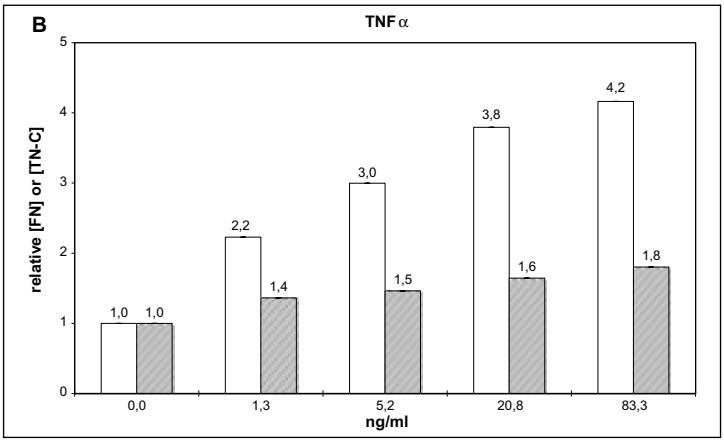
Figure 3. TNF-α and IFN-γ have a synergistic effect on tenascin-C secretion by cultured normal keratinocytes. The effect of combinations of TNF-α, IFN-γ and IL-4 in submaximal concentrations was compared to the effect of the individual components. Cytokine concentrations were 5.2 ng TNF-α per ml, 62.5 units IFN-γ per ml and 62.5 units IL-4 per ml (open bars), or 20.8 ng TNF-α per ml, 250 units IFN-γ per ml and 250 units IL-4 per ml (closed bars), respectively. The cytokines were added to confluent cultures in medium devoid of any other growth factors or cytokines. After 48 hours medium was harvested and tenascin-C concentrations were determined by ELISA. Values were normalised, and expressed relative to the tenascin-C concentration in the medium of control cultures without cytokine which was 77 ± 9 ng/ml. Data shown represent mean values of triplicate experiments ± SD.

plus IL-4, or IFN-γ plus IL-4 showed that the increase in tenascin-C protein that was induced by these combinations was comparable to the sum of the effect of the individual components. Combinations of TNF-α plus IFN-γ, however, had a much stronger effect on tenascin-C secretion than TNF-α or IFN-γ alone, suggesting that the effect of these cytokines is synergistic rather than additive (Fig. 3).

Tenascin-C and fibronectin expression in cultured keratinocytes is differentially affected by TNF-α, IFN-γ and IL-4.

In the previous section we showed that TNF-α, IFN-γ and IL-4 increase tenascin-C secretion by cultured normal keratinocytes. To investigate whether the cytokines have a general effect on secretion of extracellular matrix proteins or more specifically affect expression of tenascin-C, we quantified the concentration of another extracellular matrix protein in addition to tenascin-C. By means of a specific ELISA, fibronectin concentrations in conditioned medium of the keratinocyte cultures were determined (Fig. 4). The results indicate that keratinocytes secreted 100 to 250 times more fibronectin than tenascin-C: while tenascin-C levels were in the range of 48-77 ng/ml, fibronectin levels were as high as 6.3-





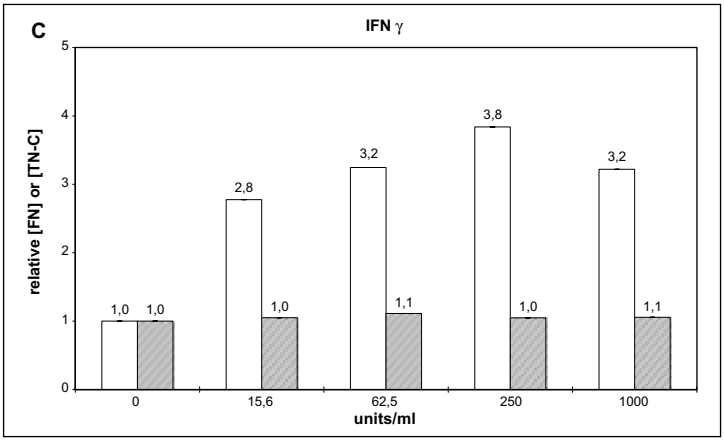


Figure 4. IFN-γ and IL-4 induce tenascin-C expression but not fibronectin expression in cultured normal keratinocytes. The effect of IL-4 (A), TNF-α (B) and IFN-γ (C) on tenascin-C secretion by normal keratinocytes was compared to their effect on fibronectin secretion. Confluent cultures were incubated with the cytokines for 48 hours. Tenascin-C (open bars) and fibronectin (closed bars) concentrations were determined by ELISA. Values were normalised, and expressed relative to the tenascin-C and fibronectin concentrations in the medium of control cultures without cytokine which were 48 ± 3 ng/ml for tenascin-C and 6.3 ± 0.4 µg/ml for fibronectin respectively.
Data shown represent mean values of triplicate experiments ± SD.

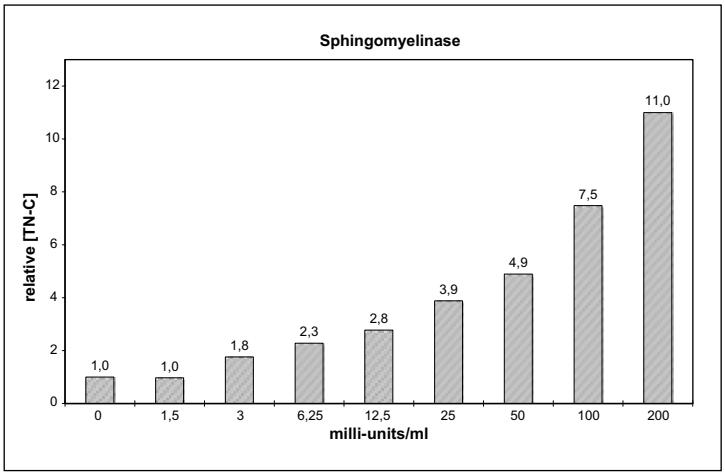
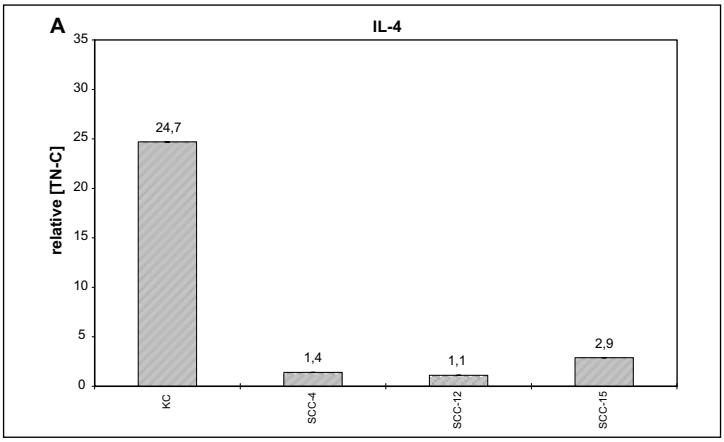


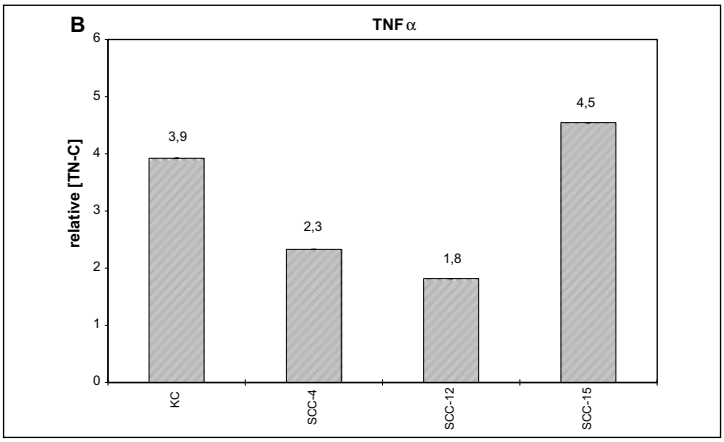
Figure 5. Sphingomyelinase increases tenascin-C secretion by cultured normal keratinocytes. Sphingomyelinase was added to confluent monolayers of normal keratinocytes. After 48 hours, medium was collected and tenascin-C concentrations were determined by ELISA. Values were normalised, and expressed relative to the tenascin-C concentration in the medium of control cultures without sphingomyelinase which was 16 ± 7 ng/ml.

23 μg/ml. TNF-α not only increased expression of tenascin-C in the keratinocytes, but also slightly increased fibronectin levels in the medium of the same cultures. At all concentrations tested, TNF-α increased fibronectin approximately half as much as tenascin-C. In contrast to TNF-α, IFN-γ and IL-4 did not affect fibronectin secretion and therefore appear to be more specific inducers of tenascin-C.

Tenascin-C expression is increased by sphingomyelinase

In an attempt to elucidate if activation of cellular stress response pathways could be responsible for the TNF-α-induced upregulation of tenascin-C expression by normal keratinocytes, we tested the effect of specific MAP kinase and MAP kinase kinase inhibitors. SB203580 and SB202190, that inhibit the p38 MAP kinase, did not significantly decrease TNF-α-induced tenascin-C secretion. No toxic effects of SB203580 and SB202190 were noted at the indicated concentrations (see table I). In addition, we tested the effect of PD98059 that inhibits the MAP kinase kinases MEK1/2, which can phosphorylate the MAP kinases p42 and p44. Addition of different concentrations of PD98059 decreased tenascin-C secretion by cultured keratinocytes to some extent. However, measurement of lactate dehydrogenase levels in the medium revealed that PD98059 had marked cytotoxic effects. Therefore, the decrease in tenascin-C levels in the presence of PD98059 may not represent a specific effect of the compound on TNF-α-induced tenascin-C expression. We have previously shown that TNF-α stimulation of keratinocytes leads to a strong increase of JNK/SAPK-1 activity (Pfundt *et al.*, Arch. Dermatol. Res., accepted). To investigate the involvement of the JNK/SAPK-1 pathway in the regulation of tenascin-C expression, we stimulated keratinocytes with sphingomyelinase, thereby gene-





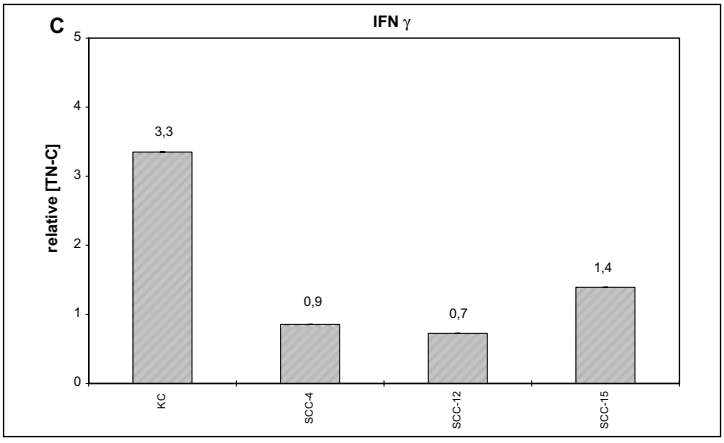


Figure 6. Regulation of tenascin-C expression by tumour-derived keratinocytes differs from the regulation in normal keratinocytes. The effect of IL-4, TNFα and IFNγ on tenascin-C secretion by the squamous cell carcinoma cell lines SCC-4, SCC-12 and SCC-15 was investigated and compared to the effect on normal keratinocytes. Stimuli were added to nearly confluent cultures. After 48 hours medium was harvested and tenascin-C concentrations were determined by ELISA. Values were normalised, and expressed relative to the tenascin-C concentration in the medium of control cultures without cytokine. Data shown represent mean values ± SD from two independent experiments for all cells except SCC-12 for which data from a single experiment are shown. Mean tenascin-C concentrations in medium without stimulus were 53 ± 17 ng/ml for normal keratinocytes, 67 ± 20 ng/ml for SCC-4, 111 ± 1 ng/ml for SCC-12 and 79 ± 17 ng/ml for SCC-15 respectively.

rating intracellular ceramide that is a known activator of the JNK/SAPK-1 pathway [307]. As illustrated in figure 5, sphingomyelinase increased tenascin-C secretion by cultured normal keratinocytes in a dose-dependent manner. To elucidate whether sphingomyelinase indeed affected tenascin-C secretion through activation of the JNKs/SAPK-1 pathway and not one of the other MAP kinase pathways, the effect of kinase inhibitors on sphingomyelinase-induced tenascin-C secretion was investigated. The effect of the p38 MAP kinase and MEK 1/2 inhibitors on sphingomyelinase-induced tenascin-C secretion was comparable to their effect on TNF-α-induced tenascin-C secretion: SB203580 and SB202190 did not significantly inhibit the sphingomyelinase-induced upregulation of tenascin-C, while the minor inhibiting effect of PD98059 again could not be discerned from its cytotoxic effects.

Tenascin-C expression by SCC cell lines is upregulated by TNF-α, but IFN-γ or IL-4 have little effect

Tenascin-C is abundant in the stroma of epidermal tumours, and previous reports suggest that part of the tenascin-C is produced by tumour cells [383-386,392]. Here, we investigated whether the squamous cell carcinoma cell lines SCC-4, SCC-12 and SCC-15 express tenascin-C. The results show that these transformed keratinocytes secrete tenascin-C into the medium when cultured in KGM or KGM/-GF. Tenascin-C levels in the medium (41-112 ng/ml) were similar to those measured for normal keratinocytes (48-77 ng/ml). To compare the regulation of tenascin-C secretion between normal keratinocytes and transformed keratinocytes, we investigated the effect of selected cytokines in KGM/-GF on tenascin-C secretion by the squamous cell carcinoma cell lines. Addition of TNF-α moderately increased tenascin-C secretion by the cell lines, but IFN-γ and IL-4 had very little effect. Results are summarised in figure 6. FCS strongly increased tenascin-C expression by the tumour cells, while it induced only a minor increase in tenascin-C levels in cultures of normal keratinocytes. Addition of 5% FCS to SCC-4 cells gave rise to tenascin-C levels that were 6.5 times the levels in control cultures without FCS. Tenascin-C levels in FCS-stimulated cultures of SCC-12 and SCC-15 cells, were 2.6 and 5.5 times the tenascin-C levels in control cultures, respectively. In contrast, normal keratinocytes produced in the presence of 5% FCS only 1.2 times the amount of tenascin-C that was measured in control cultures. Tenascin-C expression, therefore, seems to be differently regulated in the SCC cell lines compared to normal keratinocytes.

Discussion

During the last few years it has become clear that, in addition to mesenchymal cells, epithelial cells can produce tenascin-C as well. With respect to human skin, we previously showed by in situ hybridisation that tenascin-C is not expressed in the epidermis of intact normal skin, but is expressed by basal keratinocytes in the regenerating epidermis of healing wounds [377]. The results presented here confirm our previous finding that normal human keratinocytes start expressing tenascin-C when cultured in vitro [377]. This might

reflect the more general finding that keratinocytes acquire an 'activated' phenotype upon removal from their in vivo environment and subsequent in vitro culture, which phenotype resembles wound keratinocytes more than normal keratinocytes [5]. This correlates with our in situ hybridisation results showing that wound keratinocytes but not normal keratinocytes express tenascin-C mRNA in vivo and our finding that normal keratinocytes do produce tenascin-C in vitro. The basal tenascin-C production of keratinocytes in vitro is low when compared to the production by cultured skin fibroblasts and glioblastoma cell lines [377]. Yet, the results presented here indicate that tenascin-C expression can be upregulated by the inflammatory cytokines IL-4, TNF-α and IFN-γ. These cytokines have been previously reported to increase tenascin-C expression by other cell types [393-394]. Their effect on tenascin-C expression by dermal fibroblasts from healthy volunteers and scleroderma patients was recently investigated by Makhluf *et al.* [388]. They showed that IL-4 was a very potent inducer of tenascin-C production by the skin fibroblasts. TNF-α and IFN-γ had no significant effect, neither alone nor in combination. In contrast to our findings with epidermal keratinocytes, PDGF and bFGF moderately increased tenascin-C expression by the dermal fibroblasts. Together with the results presented herein, these data suggest that tenascin-C expression in skin is differently regulated for normal epidermal keratinocytes and dermal fibroblasts: while IL-4 is a strong inducer for both cell types, PDGF and bFGF only induce tenascin-C expression by dermal fibroblasts, and TNF-α and IFN-γ specifically affect tenascin-C expression by epidermal keratinocytes.

Since in skin tenascin-C is increased in conditions that are characterised by inflammation, IL-4, TNF-α and IFN-γ might be important for the increase in tenascin-C that is observed in vivo by inducing epidermal tenascin-C production. This hypothesis is supported by the fact that these inflammatory cytokines are upregulated in healing skin, a condition where keratinocytes produce tenascin-C. The dynamics of TNF-α expression in skin wounds have been investigated in detail in mice [395,396]. Experimental wounding revealed a strong and rapid induction of TNF-α during the early phase of wound healing. TNF-α was mainly produced by polymorphonuclear leukocytes but at later stages of the repair process, expression of TNF-α was also seen in macrophages and in the hyperproliferative epithelium of the wound edge. After completion of the proliferative phase of wound healing, the TNF-α expression level had returned to the basal level. The dynamics and location of TNF-α expression described in these studies correlate with the dynamics and location of tenascin-C expression in healing skin wounds [377,382]. Therefore, TNF-α might induce tenascin-C production by the wound keratinocytes. Interestingly, glucocorticoid-treatment of mice significantly reduces expression of TNF-α as well as tenascin-C during wound repair [395,397]. The results presented here suggest that a decrease in TNF-α levels in response to glucocorticoid treatment might possibly cause the concurrent decrease in tenascin-C, by reducing epidermal tenascin-C expression in the wounds.

TNF-α is known to activate cellular stress response pathways that are involved in the regulation of gene expression [307,398]. To investigate intracellular signalling events involved in the stimulation of tenascin-C expression by TNF-α, we tested the effects of specific inhibitors and an activator of MAP kinase pathways. Inhibitors of the MAP kinase p38 did not affect TNF-α-induced tenascin-C expression. Activation of the JNK/SAPK-1 pathway

by the addition of sphingomyelinase markedly increased tenascin-C expression by the keratinocytes. These results suggest that TNF-α may increase tenascin-C expression through activation of the JNK/SAPK-1 pathway. Activation of the JNK/SAPK-1 pathway ultimately leads to activation of at least two different transcription factors: ATF-2 and c-jun. Recently Mettouchi et al. showed that c-jun binds to the human tenascin-C promoter and induces transcription of the tenascin-C gene [399]. It seems likely, therefore, that both TNF-α and sphingomyelinase increase tenascin-C expression by activating the JNK/SAPK-1 pathway and subsequent transcription of the tenascin-C gene by c-jun. IL-4 and IFN-γ, the other two inducers of keratinocyte tenascin-C expression, do not affect the MAP-kinase pathways. They can regulate gene expression via another cell-signalling pathway involving different cytoplasmic protein kinases, the Janus kinases (JAKs) [400-402]. Binding of IL-4 and IFN-γ to their cell surface receptors activates specific JAKs in the cytoplasm and these JAKs subsequently activate members of the STAT transcription factor family (signal transducers and activators of transcription). These STATs activate transcription after dimerisation and binding to specific DNA sequences called gamma-interferon activation site (GAS) elements. GAS elements are defined by a small palindromic consensus sequence TTCN2-4GAA [403]. Several regions in the human tenascin-C promoter [404] conform to this consensus sequence and might therefore be regarded as putative GAS elements. We hypothesise that the induction of tenascin-C expression by IL-4 and IFN-γ that we observed in the keratinocyte cultures depends on activation of the JAK/STAT pathway by IL-4 and IFN-(and subsequent transcription of the tenascin-C gene. Since IL-4 and IFN-γ did not affect fibronectin expression by the epidermal keratinocytes, the intracellular signalling pathway that is activated by these cytokines seems not to induce expression of fibronectin.

In agreement with recent data of [392] we found that squamous cell carcinoma cells secrete tenascin-C in culture. We showed that, in addition to cells from oral squamous cell carcinomas, the cutaneous tumour cells SCC-12 express tenascin-C in vitro. Our results further indicate that inflammatory cytokines differentially affect tenascin-C expression in cultured tumour cells and normal keratinocytes. This suggests that the regulation of tenascin-C expression in skin not only differs between keratinocytes and fibroblasts, but also between normal keratinocytes and transformed keratinocytes from epithelial tumours. Tenascin-C has been implicated in the modulation of adhesion, migration and proliferation of cells. Whether the distinct regulation of tenascin-C expression that we observed in the SCC cell lines has any relevance for the behaviour of tumour cells in vivo remains to be investigated.

In conclusion, we found that TNF-α, IFN-γ and IL-4 increase tenascin-C expression by normal keratinocytes. Since in skin, increased tenascin-C expression correlates with inflammation, we hypothesise that these inflammatory cytokines might be involved in the induction of epidermal tenascin-C expression in vivo, during wound healing, malignancy, and possibly other conditions in which keratinocytes express tenascin-C.

Transcriptional regulation of the SKALP/elafin gene

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Patrick Zeeuwen
Henri Molhuizen
Joost Schalkwijk

Abstract

Keratinocytes of inflamed epidermis (psoriasis, wound healing, irradiated skin) are hyperproliferative and display an abnormal differentiation program. This so-called regenerative maturation pathway is characterised by the induction of a set of genes that is not expressed by keratinocytes in normal skin, such as the cytokeratins CK6, CK16 and CK17, and the proteinase inhibitor SKALP/elafin. In a previous study we showed that in cultured keratinocytes, SKALP expression can be induced by the addition of FCS or TNF-α to undifferentiated primary keratinocytes, and that this induction depends on the activity of the P38 MAP kinase. Here we have studied the regulation of SKALP/elafin gene expression in epidermal keratinocytes at the molecular level as a paradigm for the regenerative maturation pathway. We have constructed deletion mutants of the SKALP/elafin promoter region that conferred keratinocyte-specific expression upon transient transfection. Unfortunately the promoter-constructs were not subjected to identical regulation mediation as the endogenous gene since they were highly active, independent of stimulation by TNF-α or FCS. A two step deletion of a region that contained several potential NF-IL6 binding sites and two NFκB binding sites resulted in a 90% reduction of promoter activity. However upon the induction of the endogenous SKALP gene expression by either FCS or TNF-α, a specific and quick translocation of the transcription factor NFκB in the nuclei of the keratinocytes was observed. Transcription factors AP-1 and NF-IL6 were found to be constitutively present in the nuclei of both resting and activated keratinocytes. These findings suggest that SKALP gene expression could be regulated by a NFκB dependent mechanism. We speculate that SKALP expression by keratinocytes is part of an epidermal stress response pathway which is characteristic for e.g. psoriatic epidermis and healing skin wounds and in which NFκB and p38 are likely to play crucial roles.

Introduction

Human epidermis is mainly composed of keratinocytes, which follow a highly co-ordinated process of terminal differentiation. This normal epidermal differentiation pathway ultimately leads to the formation of a protective layer of dead corneocytes [259,260]. There are however circumstances, e.g. wounding and inflammation, in which the epidermis is forced to follow an alternative pathway. This alternative pathway, that is characterised by hyperproliferation and alternative differentiation, is a functional physiological response to conditions that require rapid epidermal regeneration and protective gene expression like e.g. wound closing. This pathway is generally referred to as regenerative maturation [6,7]. There are however a number of skin conditions where this pathway seems inappropriate and is part of a pathological skin condition, like e.g. psoriasis [10]. There is not much known about the molecular changes that are at the basis of this change in epidermal differentiation pathways. Insight into these earliest changes at the molecular level could provide us with knowledge about the regulation of epidermal differentiation routes, and could ultimately lead to the identification of novel targets for the therapy of skin diseases.

In this study we present data derived from a number of experiments in which we have studied the expression regulation of the serine proteinase inhibitor SKALP (Skin Derived AntiLeuko- Proteinase) at the molecular level. Similar to the expression of the cytokeratins 6,16 and 17 [12,13], the expression of the SKALP gene is tightly correlated with abnormal epithelial differentiation in a context of hyperproliferation. SKALP is highly expressed in psoriatic epidermis [255,277] and is inducible *in vivo* by tapestripping (standardised model for epidermal injury and trans-epidermal water loss) [202,204] and UV-B irradiation (Pfundt *et al* in submitted). SKALP expression is therefore a model for gene expression that is characteristic for the epidermal phenotype generally referred to as regenerative maturation. Assuming a general mechanism, it is our belief that knowledge on the molecular regulation of SKALP expression should be informative on the molecular regulation of the epidermal phenotype of regenerative maturation. In previous studies we have shown that SKALP gene expression can be induced *in vitro* by subjecting cultured undifferentiated primary keratinocytes to either FCS or TNF-α [77,294] (Pfundt *et al* in press). This in vitro induction of SKALP was shown to be dependent on the expression of the activity of the MAP kinase family member p38. In addition we were able to show that SKALP expression *in vivo* induced through UV-B irradiation of normal human skin, is also correlated with p38 activation (Pfundt *et al*, submitted). This led to the hypothesis that the expression of SKALP is part of an epidermal stress response that is aberrantly induced and maintained in pathological skin conditions like psoriasis. We were therefore interested to investigate the regulation of SKALP gene expression at the molecular level.

By determining the transcription initiation point of the SKALP gene we show that the region which was presumed to be the SKALP promoter region, indeed is the promoter region of the SKALP gene. Transcription initiation can occur at multiple sites in a region 10-24 bases upstream of the ATG startcodon, resulting in a very short 5'-untranslated region. We demonstrate the specificity of SKALP promoter activity with respect to cell type. In addition we also demonstrate that induction of SKALP expression is accompanied with the presence of high levels of activated transcription factor NFκB in the nuclei of the stimulated keratinocytes, whereas AP1 and NF-IL6 levels remain unchanged. Promoter/reporter constructs that comprised 4500 bp of upstream and 1600 bp of downstream SKALP gene sequence (relative to translation start-site) were analysed in transient transfection experiments in keratinocytes under various culture conditions. These experiments showed that the promoter/reporter constructs are highly active under all culture conditions in contrast to the endogenous SKALP gene. This discrepancy between the promoter/reporter constructs and the endogenous SKALP gene suggest regulation of SKALP gene expression at other levels like chromatin structuring and/or methylation. We speculate on the importance of the NFκB activation, the potential consequences of the short 5'-untranslated region of mRNA derived from SKALP gene expression and on the potential mechanisms involved in the regulation of the SKALP gene.

Material and methods

All chemicals were obtained from Merck (Merck, Darmstadt, Germany) unless specified differently. Restriction enzymes were provided by Boehringer (Boehringer, Mannheim, Germany). Primers were provided by Isogen (Isogen, Amsterdam, the Netherlands).

Isolation and cloning of the human SKALP gene promoter region in/construction of reporter gene constructs

We have previously reported the cloning of a 9kb *Hind*III genomic fragment in pBluescript (Stratagene, USA) which was characterised as containing the human SKALP gene [257]. This clone was designated pSKAG. From this clone an *Eco*RI/*Xba*I fragment containing approximately 1-kb promoter region in addition to a small part of the first exon was cloned in pBluescript KS^+ resulting in clone pSPRO29. Construct pSPRO29 was used as template in a PCR reaction synthesising the 1 kb promoter fragment (pos. -1012/+13; all nucleotide positions in this paper are given relative to the start site of translation (see figure 1)). Two primers (forward: 5'-GAATTCCCGGGTTCATATAT-3' (pos.-1012/-993, (mutated nucleotide is underlined)), reverse: 5'-TGCTGGCCCCCATGGTGTCA (pos. -7/+13)) were used to introduce a *Sma*I site and a *Nco*I at the 5' and the 3' site of the PCR product respectively. The PCR-product was cloned in the pGEM-4 T-vector (Promega, Madison, USA) and sequenced for verification. From this clone a 1-kb *Sma*I/*Nco*I fragment (pos. -1004/+3) was directionally subcloned in frame with the firefly luciferase gene in pSla4, a modified pSla3 vector [405], directly fused to the ATG of the luciferase gene through the *Nco*I site. The resulting clone was designated pSPL1000. From this clone deletion mutants were derived, designated pSPL440, pSPL290, pSPL170 and pSPL130. All these constructs were prepared according to standard cloning techniques using available restriction sites, except clone pSPL130. To generate this clone we used a mutagenic forward primer (5'-GTGGATATCTCCCAATAC-3' (pos. -132/-115)) in a PCR reaction with the previously mentioned reverse primer to introduce an *Eco*RV site at position -126. From the resulting PCR product an *Eco*RV/*Nco*I fragment was cloned in a *Sma*I/*Nco*I digested pSLA4 vector. The largest transfection construct pSPL4500 was constructed via directional cloning of an approximately 4 kb *Hind*III/*Bgl*II promoter fragment (pos. -4500/-435) in a *Sma*I/*Bgl*II digested pSPL1000 construct. The two promoter constructs pSPL1000int+ and pSPL1000int- contain the entire genomic sequence of the SKALP gene in the correct and reverse orientation respectively. Both constructs containing the genomic SKALP sequence were constructed by directional cloning of a PCR product containing the entire gene sequence (pos. +3/+1631) in the *Kpn*I site of construct pSPL1000 between the stop codon and the first polyadenylation signal of the luciferase gene. Finally a construct containing the insert from clone pSPL1000 in the reverse orientation, designated pSPL1000rev was generated. To obtain this clone, the previously mentioned 1 kb *Sma*I/*Nco*I fragment was used. A blunt end was generated at the *Nco*I site, and the complete fragment was subcloned in the *Sma*I site from pSla4, thus maintaining the original translation start codon from the luciferase gene. Figure 2 gives a schematically representation of these 9 constructs.

pSPL1000⇓ NFκB

-1012 GAATTCCCTG GTTCATATAT ATCACCTTTT CCAGAAGAAG GAACCCCCGT TTTCCCCTTT

-952 CATTACTCAT TCAAATAAAA TATGTTAATA GACCAGACCA AGTCTCACAC ACAACACACA

-892 CACACACACT CACACACACA CACACACACA CACACCCCTA CCTGTGGTCA GGGGTCAGGC

-832 TTTCATGATT AGCAGTCCCC ATTAGACTCA CAAGTTGATG GTTCAAAGAG ACAACAATTC

-772 TTCACAAGAA ACAGGGTGCT GTTCAAAGAA GTGGGGAGGC ATGCTGGACA CACACACTGT

-712 AGATGTCCAA TGTATACATG ATACATGTTT TCTACTACTT TCTGATTATT TTCTCCCTAC

-652 CACTGTGATT TCAAGGGCAT CATCCCTCTT CAGACCTAGA ATTCCACCTT CCAATTACTT

AP-1

-592 TCTTGATGCA TCTGAAAGGC CGTCTCTGAA ACAGCAAAGT GCAATAATTA GTCATTATGT

-532 ATTCAAGGCA AATTTGTCTT ACAGAGTTTT TTGCAGGACC AGGGAAGAAG GAAGGAAATG

pSPL440⇓

-472 CCCAGTTTGA TGCTGGGAGT GGTAAAATGA TAAAGTAGAT CTGGGTGGGG TTTGTAGCAC

NF-IL6 NF-IL6

-412 CAGAGCATAA TGGAGAAACA CCTTGGTTTT GTAATCAAGA CTGGATCTAC CAGTGACTTG

NF-IL6/AP-1 NF-IL6

-352 CTGAATAACC TTCGGTGATT CCTTTCTCTT CTTGGGTCTC ACTGTATTTC AAAACATGAA

SPL290⇓

-292 GAATTTCATT GTAATGTTAC CTAATAAGTG AGCCAGCACT TCTACTCTGT GAGAAAGTAG

NF-IL6 pSPL170⇓ AP-1

-232 GAAAACTCTT GGGACAATCA GAGATGATGT GATGTAATGT CCATTAGTTC TTCCTGTGAA

AP-1 NFκB pSPL130⇓ NF-IL6

-172 TAATCCTGAG GGAAAGCCCC CAGGTCCCTC CCAGAATGGG GTGGATATTT CCCAATACAG

SP1

-112 CTAAGGAATT ATCCCTTGTA AATACCACAG ACCCGCCCCT GGAGCCAGGC CAAGCTGGAC

-52 TGCATAAAGA TTGGTATGGC CTTAGCTCTT AGCC*AAA*CAC CTTCCTGACA CCATG

+1

Figure 1. Nucleotide sequence of the human SKALP gene promoter region. Numbering is relative to the first base of the ATG translation codon (printed in bold). The borders of the fragments used for the luciferase transfection constructs are indicated with arrows (⇓). The putative 'CCAAT' and 'TATA' boxes are double underlined. The region of multiple transcription start sites is underlined with a dotted line, the preferential transcription start sites are printed in italics and bold. Consensus binding sequences for known transcription factors are indicated by name and underlined.

Cell cultures

Keratinocytes were seeded in 6 well culture dishes and cultured until confluence in keratinocyte growth medium (KGM) as described before [77]. KGM was composed of keratinocyte basal medium (KBM (Biowhittaker, Walkersville, Maryland, USA); 0.15 mM Calcium), supplemented with ethanolamine (0.1 mM) (Sigma, St.Louis, USA), phosphoethanolamine (0.1 mM) (Sigma, St.Louis, USA), bovine pituitary extract (BPE; 0.4 $\%^{v}/_{v}$) (Biowhittaker, Walkersville, Maryland, USA), insulin (5 (g/ml), (Sigma, St. Louis, USA), hydrocortisone (0.5 µg/ml) (Collaborative Research Inc.), Recombinant Mouse

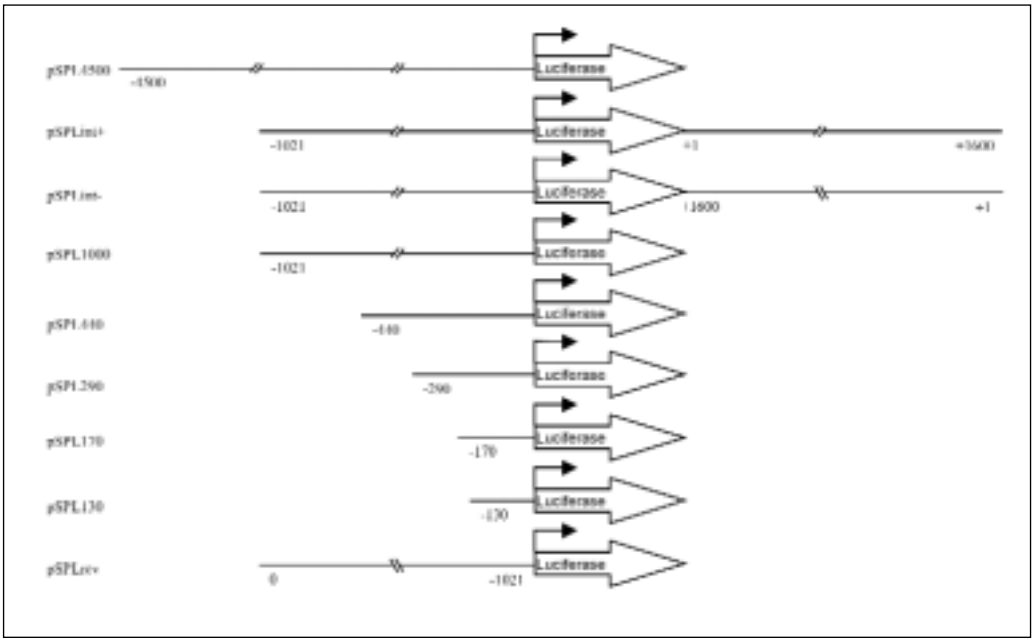

Figure 2. Schematic representation of the luciferase constructs containing deletion fragments from the promoter region of the SKALP gene. Nucleotide positions are given relative to the translation start codon and indicate the orientation of the deletion fragment.

Epidermal Growth Factor (EGF; 10 ng/ml) (Sigma, St. Louis, USA), penicillin (100 U/ml) (Gibco, Breda, the Netherlands) and streptomycin (100 µg/ml) (Gibco, Breda, the Netherlands). At confluence the cells were either transfected or switched to one of the test media, subsequently the growth factors or cytokines that were to be tested were added in different concentrations. Induction of keratinocyte differentiation was established by switching the cells at confluence either to KGM supplemented with 5 $\%^{v}/_{v}$ FCS (KGM/FCS) (FCS from Seralab, Nistelrode, the Netherlands) or to KGM depleted of growth factors (BPE, Insulin and EGF) and hydrocortisone (KGM-GF) [265]. Human skin fibroblasts derived from healthy volunteers, glioblastoma cells and A431 cells were seeded and cultured in 6 well culture dishes in A1 medium (DMEM/F12 (Flow Laboratories, Irvine, Scotland) in a ratio of 3:1 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10 $\%^{v}/_{v}$ FCS). At 50-70 % confluence the cells were transfected.

Transfections

Keratinocytes were transfected at confluence. Prior to transfection the cells were washed twice with 1 ml KBM and 900 µl KBM was added. Transfection mixes were made containing 0.75 pmol transfection construct 0.5 pmol EF2-βGAL normalisation construct (a kind gift from Dr. G.Swart, department of Biochemistry, University of Nijmegen) and 25 µl lipidfection reagent (lipidfection reagent consisted of 0.8 mM DDAB (di-methyldioc-

tadecylammonium bromide (Sigma, St. Louis, USA), and 1.7 mM PtdEtn (dioleoyl-L-α-phosphatidylethanolamine (Sigma, St. Louis, USA), prepared according to [406]) in a final volume of 100 µl KBM. These mixes were incubated for 20 minutes at room temperature, and added drop-wise to the cells, resulting in a final transfection volume of 1 ml. After 6 h of incubation 1 ml KGM was added to the cells. Twenty-four hrs. after transfection the medium was aspirated and the cells were switched either to KGM/FCS or to KGM-GF, and incubated for another 24 h. after which the cells were harvested.

The human fibroblasts, glioblastoma cells and A431 cells were transfected at 50-70 % confluence. Prior to transfection the cells were washed twice with 1 ml DMEM/F12 (ratio 3:1) and 900 µl DMEM/F12 was added. Transfection mixes were made containing 1.3 pmol transfection construct, 0.5 pmol EF2-βGAL and 25 µl DOSPER (Boehringer, Mannheim, Germany) in a final volume of 100 µl DMEM/F12. These mixes were incubated for 20 minutes at room temperature and added drop-wise to the cells. After 6 h of incubation 1 ml A1 medium was added to the cells. Twenty-four h after transfection the medium was aspirated and two ml A1 was added. After a final incubation of 24 h the cells were harvested.

Cell harvesting and Luciferase and β-Galactosidase assays

Transfected cells were washed twice with PBS (NPBI, Emmer-Compascuum, the Netherlands) and lysed in 150 µl reporter lysis buffer (Promega, Madison, USA). Luciferase enzyme assay was performed with the Luciferase Assay System (Promega, Madison, US) as suggested by the manufacturer using 10 µl cell lysate and 50 µl assay reagent. Beta-galactosidase enzyme assay was performed with the 'Galacto-Light Plus' chemiluminescent reporter assay for the detection of β-galactosidase (Tropix, Bedford Massachusetts, USA) as suggested by the manufacturer using 20 µl cell lysate. Promoter activities were measured as the ratio between the luciferase activity and β-galactosidase activity per sample.

Determination of SKALP mRNA transcription start-site using S1-nuclease mapping

S1 nuclease mapping using a single stranded DNA probe was used to determine the start-site of transcription of the SKALP gene. To obtain a suitable DNA probe we cloned a *Bgl*II/*Xba*I fragment (pos. -435 to +62) in a *Xba*I/*Bam*HI digested pBluescript KS^+ vector. This subclone was used for transformation of XL1-Blue cells (Stratagene, USA). From a single XL1-Blue colony containing the appropriate clone 2 ml of TYP/Amp broth (16 g Bacto-tryptone, 16 g Bacto-yeast extract, 5 g NaCl and 2.5 g K_2HPO_4 per liter with ampicillin (50 mg/ml)) was inoculated and cultured for 16 h (37˚C, 250 rpm). The next day 100 µl of this culture was used to inoculate 5 ml TYP/Amp broth. After 30 minutes 40 µl of a M13K07 helper phage stock ($5x10^{10}$ plaque forming units) was added, and the culture was grown overnight. The following day single stranded DNA was isolated using standard procedures. To prepare the radiolabeled probe, T7 primer (Promega, Madison, USA) was annealed to 250 ng single stranded DNA, and primer extension was performed with 5 units of T7 DNA polymerase (Pharmacia, Upsala, Sweden). The primer extension was performed in a 30 µl reaction containing 0.5 µM [α-^{32}P]-dCTP (3000 Ci/mmol, (Amersham,

Buckinghamshire, England)), 0.4 mM dATP, 0.4 mM dTTP, 0.4 mM dGTP and 1.3 µM dCTP (Pharmacia, Upsala, Sweden). After 15 minutes incubation at 37˚C, 2 µl 10 mM dCTP and 5 units T7 DNA polymerase were added. The reaction was terminated by incubation at 65˚C for 10 minutes. This inactivation was followed by digestion with *Eco*RI for 2h at 37˚C in order to remove the insert. After digestion the DNA was denatured and separated on a 4 % polyacrylamide gel. The radiolabeled probe was isolated from this gel.

Total RNA isolated from keratinocytes grown in the presence of 5 % FCS was used as a source for SKALP mRNA. Fifty µg of this total RNA was mixed with $8x10^5$ cpm single stranded probe DNA, precipitated, and washed once with 70 % ethanol. As a negative control Yeast tRNA (Boehringer, Mannheim, Germany) was used. The pellets were dissolved in 30 µl hybridisation buffer (40 mM PIPES (piperazine-N,N,-bis[2-ethanesulfonic acid]) pH8.0, 0.5 M NaCl, and 80 % formamide) and incubated for 10 minutes at 85˚C followed by an incubation for 16 h at 50˚C. After this probe hybridisation, 300 µl nuclease S1 reaction mix (280 mM NaCl, 5 mM NaAc pH4.5, 4.5 mM $ZnSO_4$, 20 (g/ml herring sperm DNA (Boehringer, Mannheim, Germany) and 1000 U/ml Nuclease S1 (Pharmacia, Upsala, Sweden)) was added and the reaction was incubated at 37˚C for 60 minutes. As a control reaction to check for sufficient nuclease S1 activity, free radiolabeled probe was subjected to nuclease S1 digestion. The nuclease S1 reactions were terminated by addition of 80 µl stop buffer (4M NaAc, 50 mM EDTA pH8.0 and 50 (g/ml yeast tRNA), followed by ethanol precipitation. The pellets were washed with 70 % ethanol, dried, resuspended in 3 µl H_2O and incubated at 65˚C for 5 minutes. After this incubation 4 µl formamide loading dye (95 % Formamid, 10 mM EDTA, 0.1 % bromephenol blue and 0.1 % xylene cyanol) was added, and the samples were boiled for 4 minutes and chilled on ice to denature the DNA. The samples were separated on an 8 % polyacrylamide gel, and autoradiography was performed using Kodak X-Omat 100 X-ray film.

Determination of SKALP mRNA transcription start site using 5'-RACE

Rapid amplification of cDNA ends (RACE) was used to reconfirm transcription start sites found with S1-nuclease mapping. Using the 5'/3'-RACE kit (Boehringer, Mannheim, Germany) we performed 5' RACE on 10 (g of total RNA isolated from keratinocytes grown in KGM/FCS, as suggested by the manufacturer. In the RACE reaction the SKALP specific primers (SP) used were the primers SP1 (+1013/+1037), SP2 (+942/+966) and SP3 (+30/+48). The produced RACE fragment was purified on an agarose gel and cloned into the pGEM-T vector (Promega, Madison, USA). The RACE clones obtained were sequenced by means of the dideoxy chain termination method.

Preparation of nuclear extracts

At different time points after the stimulation of confluent primary human keratinocytes the cells were harvested for nuclear protein extraction. Nuclear extracts were prepared as described by Scott *et al* [407]. In short, approximately 3-6 10^6 cells were washed with ice cold PBS and harvested by gently scraping using a rubber policeman and collected in ice cold buffer A (10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT). This cell suspension was incubated on ice for 15 min allowing the cells to swell in the hypotonic solution.

Table 1 Overview of oligonucleotides that were used for EMSA analyses

Transcriptionfactor	Strand	Sequence of oligonucleotide
NFκB	Upper	5'-AGTTGAGGGGACTTTCCCAGGC-3'
	Lower	3'-TCAACTCCCCTGAAAGGGTCCG-5'
AP1	Upper	5'-CGCTTGATGACTCAGCCGGAA-3'
	Lower	3'-GCGAACTACTGAGACGGCCTT-5'
NF-IL6	Upper	5'-TGCAGATTGCGCAATCTGCA-3'
	Lower	3'-ACGTCTAACGCGTTAGACGT-5'

Subsequently Nonidet NP-40 solution was added to the swelled cells to a final concentration of 0.2 %. The keratinocytes were lysed in a dounce homogeniser by 20 strokes with pestle B. The nuclei were pelleted by centrifugation (30 sec. 13000 rpm) and the nuclear pellet was resuspended in 150 µl ice cold buffer C (20 mM HEPES, 10 % glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM EDTA, 1 mM PMSF). This nuclear suspension was shaken vigorously on a shaking platform at 4˚C for 10 minutes, after which the nuclear fragments were pelleted by centrifugation (5 min, 13000 rpm). The supernatant containing the nuclear proteins was aliquoted and stored at -70˚C. One aliquot was used for protein quantification by means of a colorimetric assay (Bio-Rad DC Protein Assay Kit, BioRad).

Electrophoretic mobility shift assay (EMSA)

For the protein-ODN binding reaction, 10^5 cpm of ^{32}P-labeled ODN, 1 µg of non-specific competitor DNA poly(dI-dC) (Pharmacia Biotech, Upsala, Sweden), and 10 µg nuclear protein extract were added to a buffer containing 10 mM Tris pH7.6, 50 mM KCl, 1 mM EDTA, 5 % glycerol, and 1 mM DTT. This mixture was incubated for 30 minutes at room temperature. Reaction mixture was run on a 4 % non-denaturing polyacrylamide gel at 10-15 Volts/cm during 2 h. Gels were dried on a gel dryer (Savant model SGD2000) and autoradiographed at -80˚C using X-Omat X-ray films (Kodak) at -80˚C with an intensifying screen. The specificity of the protein-DNA interaction was checked by the addition of a 10-50 molar excess of unlabeled double stranded oligonucleotide to the binding reaction. In order to investigate the potential involvement of a particular protein in a specific DNA protein interaction, supershift experiments were performed using a rabbit polyclonal antibody directed against the NFκB p65 subunit (SantaCruz, Santa Cruz, CA, USA) as follows. The nuclear protein extracts were preïncubated with different concentrations of the antibody for 10 min at roomtemperature before radiolabeled oligonucleotide was added. The total mixture was incubated for another 20 min before loading on the gel.

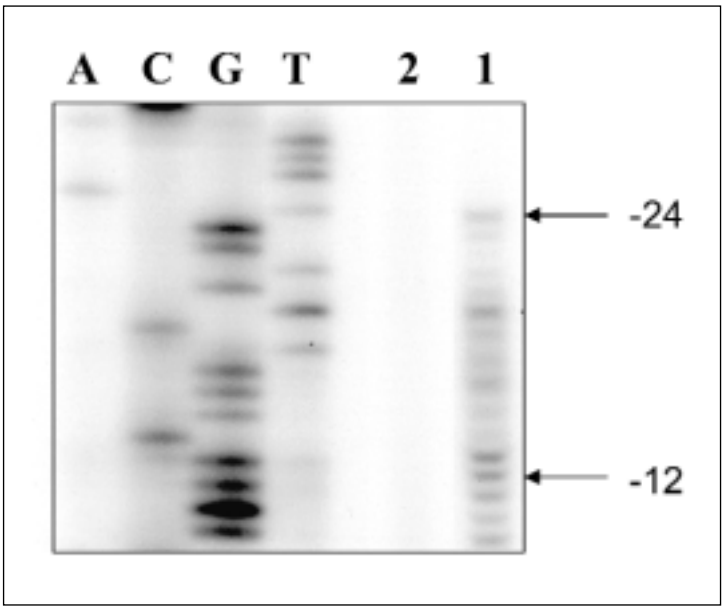

Figure 3. Determination of the region where transcription of the SKALP gene initiates. A number of major transcription startpoints are indicated at the right side of the figure with nucleotide numbers (relative to translation startsite) corresponding with bands found in S1 treated keratinocyte RNA (lane 1). In the control lane, which was loaded with S1 treated tRNA no bands are visible confirming specificity (lane 2). At the left side of the figure a sequence pattern is visible that was used as a molecular weight marker.

Results

The human SKALP gene contains multiple transcription start sites

The cloning and partial structural characterisation of the SKALP gene and cDNA has been described previously [256,257,408]. Based upon the presence of consensus sequences for a TATA and CCAAT box (see figure 1), the region directly 5' of the translation start site was assigned as the putative promoter. In order to verify this region as being the SKALP promoter, the S1-nuclease mapping technique was used to determine the transcription start site of the SKALP gene. Using this technique, multiple transcription start sites were found 13 to 24 basepairs 5' of the translation start site of the SKALP gene (figure 3). Identical results were found at different hybridisation temperatures (not shown). Since these positions would result in an unusually short 3' untranslated region (3'UTR), an independent method was used to confirm this region as being the region where SKALP transcription can start. Using the 5' RACE technique we found identical results (see figure 4). Figure 4 also shows that although multiple transcription start sites have been identified there seems to be a preference for the positions -16, -17, and -18. Sixty percent of the RACE clones that were analysed started at one of these nucleotides (see figure 4).

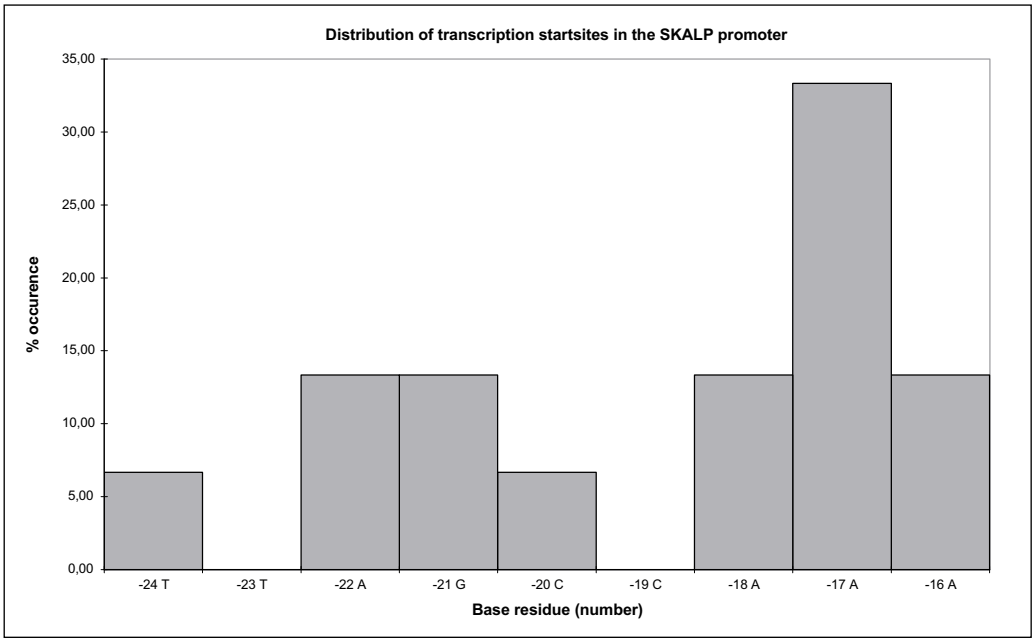

Figure 4. Relative distribution of transcription start sites in the SKALP gene promoter determined by 5'-RACE. Nucleotide positions (relative to the translation startsite) are indicated on the X-axis. In the promoter region between -16 and -24, transcription can initiate at several positions. However there seems to be a preference for the position -16/-17/-18. These results confirm the region found using nuclease S1 mapping (figure 3).

The region between -290 and -440 basepairs 5' of the translation startsite of the SKALP gene is important for promoter activity in human keratinocytes

In order to scan the SKALP promoter for sequences relevant for transcriptional activity in human keratinocytes, a set of 9 reporter constructs was designed covering approximately 4500 basepairs of upstream and approximately 1600 basepairs of downstream gene sequences for testing in transiently transfected human keratinocytes (see figure 2). Because of previous reports describing side effects of lipidfection on keratinocytes [409] we verified that the transfection procedure as such did not induce any endogenous SKALP expression in cultured human keratinocytes. Stepwise deletion of the region between -4500 and -440 did not influence SKALP promoter activity, as can be seen by the relative luciferase activity of the transfection clones pSPL4500, pSPL1000, and pSPL440. Based on the relative luciferase activity of the transfection clones pSPL1000int+ and pSPL1000int-, containing the intron sequences of the SKALP gene in both orientations, it appears that the intron sequences do not have any effect on promoter activity in either orientation. However deletion of the region -440 and -290 (clone pSPL290) resulted in a dramatic decrease of SKALP promoter activity in human keratinocytes of about 75 % (see figure 5). Further deletion of promoter sequences down to 170 basepairs 5' of the translation start site (pSPL170) resulted in a further decrease of promoter activity down to 10 % (see figu-

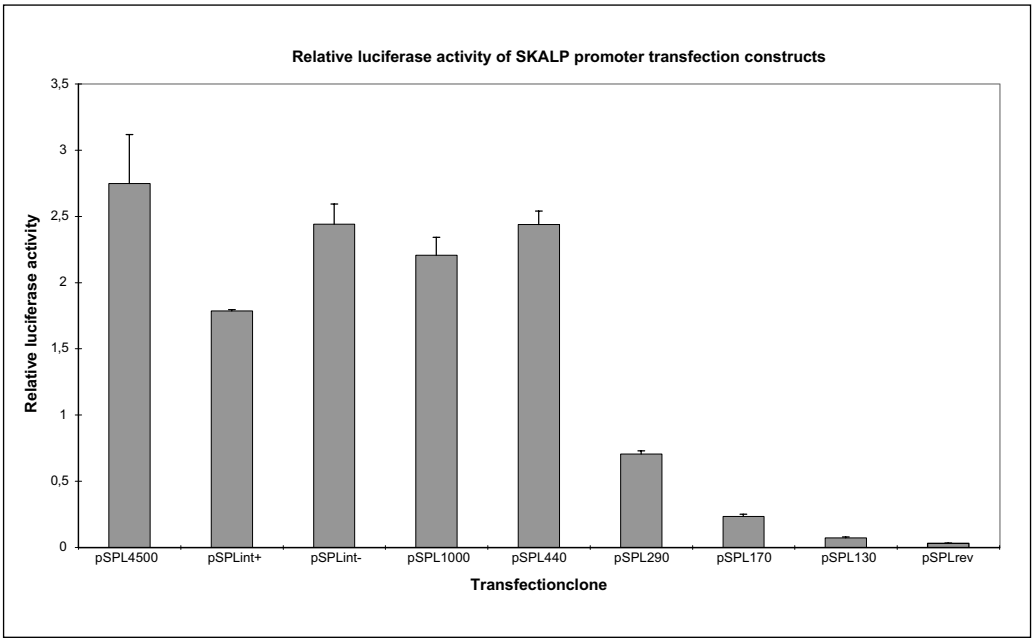

Figure 5. Relative promoter activity of the various SKALP promoter reporter constructs, defined as the relative luciferase activity. The bars represent the relative luciferase activity found in cell lysates of transfected keratinocytes cultured in KGM/FCS. The error bars indicate the standard error of the mean of three independent transfection experiments. Deletion of the sequence region between -4500 and -440 (pSPL4500/pSPL1000/pSPL440) does not affect promoter activity. Intron sequences have virtually no effect in either orientation (pSPLint+/pSPLint-). Deletion of the region between -440 and -290 (pSPL290) results in a dramatic loss of promoter activity of 75%. Upon further deletion of promoter sequences (pSPL170/pSPL130), the promoter loses all activity. The negative construct pSPLrev has no significant promoter activity.

re 5). Transfection clone pSPL130, containing only minimal promoter consensus binding sequences showed almost no promoter activity in keratinocytes. Clone pSPL1000rev, in which the upstream sequence of the SKALP gene is cloned in reverse orientation, resulted in a luciferase activity comparable to the empty luciferase gene vector pSLA4 (results are shown in figure 5). Figure 5 shows the activity of the various promoter constructs transfected in keratinocytes that were subsequently induced to differentiate in KGM with foetal calf serum (KGM/FCS). FCS also induces expression of the endogenous SKALP gene [77]. When the transfected cells were induced to differentiate by growth factor depletion (KGM/-GF), under which condition the endogenous SKALP gene is only expressed at low levels, also high levels of the luciferase constructs were found, although at somewhat lower levels than in KGM/FCS (not shown). These findings suggest additional regulatory mechanisms for expression of the endogenous gene compared to the promoter region used in this study, as will be discussed below.

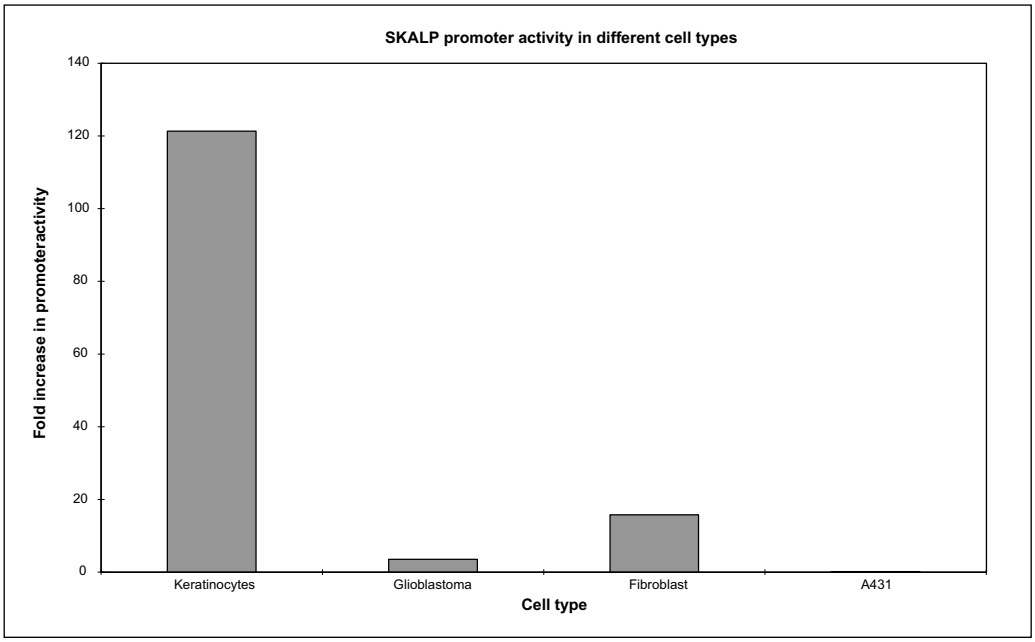

Figure 6. SKALP promoter activity in different cell types. The bars represent the promoter activity of transfection clone pSPL1000 compared to the empty reporter construct pSLA4 (assigned a value of 1). In keratinocytes cultured in KGM/FCS, the SKALP promoter shows high promoter activity. However in the glioblastomas, the fibroblasts, or in the A431 cells the promoter shows almost no activity.

The SKALP promoter confers keratinocyte specific gene expression

To investigate whether the promoter region that was used in this study could confer cell-type specific expression, reporter constructs were transfected in human fibroblasts, glioblastoma cells, and A431 cells. Figure 6 shows the relative activity of the transfection clone pSPL1000 in the different cell types, compared to the relative activity of the empty luciferase gene vector pSLA4. As can be seen in figure 6, the SKALP promoter was highly active in keratinocytes, cultured in KGM/FCS. Whereas in fibroblasts, glioblastoma cells, and in A431 cells the SKALP promoter showed hardly any transcriptional activity. This effect was also observed with other transfection constructs (data not shown).

In contrast to AP1 and NF-IL6, the transcription factor NFκB is activated upon the induction of SKALP expression.

Analysis of the promoter region of the SKALP gene reveals potential binding sites for transcription factors that are known to be involved in early stress responses, such as AP-1, NF-IL6 and NFκB (see figure 1). This is compatible with the idea that induction of SKALP expression is part of an epidermal stress response (see chapter 3.1). In order to investigate the activation and presence of these transcription factors in the nuclei of keratinocytes that are induced to express SKALP, we have performed a number of electroforetic mobili-

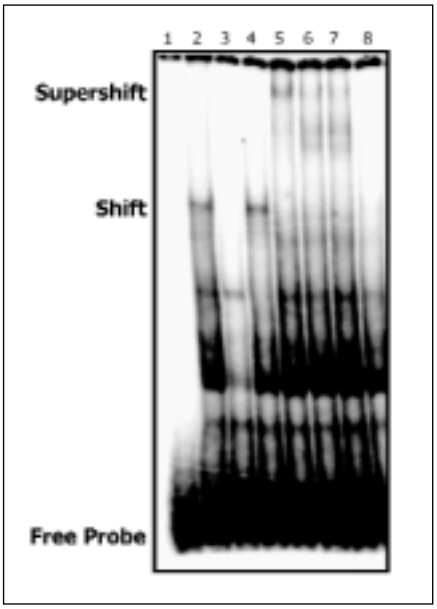

Figure 7. Electrophoresis mobility shift analysis of nuclear extracts from keratinocytes combined with labeled double stranded oligonucleotide comprising a NFκB consensus motif (see table 1). The lanes correspond with EMSA's using NFκB probe combined with extract from keratinocytes Lane1: no extract added, lane 2: nuclear extract after 1.5 hrs with 5 % FCS, lane 3: as lane 2 with 10 times excess cold specific probe, lane 4: as lane 2 with 10 times excess non-specific cold probe, lane 5: as lane 2 preïncubated with 1 μg NFκB antibody, lane 6: as lane 2 preïncubated with 0.1 μg NFκB antibody, lane 7: as lane 2 preïncubated with 0.04 μg NFκB antibody, lane 8: nuclear extract after 1.5 hrs without FCS.

ty shift assays (EMSAs or bandshifts). In these EMSAs we have used probes that contain consensus-binding sequences for either AP1, NF-IL6 or NFκB (see table 1) combined with nuclear extracts of keratinocytes that were harvested at different timepoints after the induction of SKALP expression (1, 8, 24 and 48 hours after induction). These transcription factors all have multiple potential binding sites in the SKALP promoter (see figure 1) and could be relevant for the regulation of its expression. Moreover there have been a number of papers that found the relevance of these factors in the regulation of keratinocyte gene expression in general or [326,410], as is the case for AP1, for the regulation of SKALP expression in particular [301,330]. We found that upon the induction of SKALP expression by either FCS or TNF-α, within one hour the transcription factor NFκB is activated and translocated to the nucleus (see figure 7). After serum stimulation NFκB can be found in the nucleus until at least 8 hours after stimulation. Twenty four hours after the induction of SKALP using serum we could no longer detect NFκB in the nuclei. This in contrast to TNF-α where NFκB remained in the nuclei until at least 48 hours after stimulation. In unstimulated keratinocytes (KGM-gf or KGM) no activated NFκB could be found in the nuclei of the cells. The specificity of this binding reaction was confirmed with competition experiments. Moreover, supershift experiments using an antibody directed against the p65 subunit of the NFκB transcription factor complex confirm the involvement of NFκB in the mobility shifts that we had seen (see figure 7). This antibody was also used to reconfirm NFκB translocation upon TNF-α and FCS addition immunocytochemmically. These experiments gave identical results (data not shown).

We also performed EMSA experiments using nuclear extracts identical to those used in the NFκB experiments combined with oligonucleotide probes harbouring consensus bin-

ding sequences for the transcription factors AP1 and NF-IL6. For both AP1 and NF-IL6 we observed identical mobility shifts in all the nuclear extract samples. However, no differences were found between nuclear extracts derived from cells that were or were not induced to express SKALP at any timepoint after induction (data not shown).

Discussion

Analysis of wounded epidermis or epidermis affected by various diseases (including neoplasia) reveals a gene expression pattern that is different from that found in normal unaffected epidermis. A large number of genes show changes in the level of gene expression or in their spatial regulation with respect to stratum specificity. Beside these genes there is also a group of genes that is not expressed in normal skin but is specifically *de novo* expressed in disturbed epidermis. To this group of genes belong structural proteins like the cytokeratins CK6, CK16 and CK17, also referred to as hyperproliferation- or disease-associated keratins [12,13,200]. It should however be emphasised that these keratins are not specific for hyperproliferative epidermis. They are also found in various non-epidermal keratinocytes undergoing hyperproliferation and are considered as markers for hyperproliferative keratinocytes in general [270]. In addition to these structural proteins also the proteinase inhibitor and antimicrobial [355] protein SKALP is *de novo* expressed under these abnormal epidermal circumstances. We have shown in an earlier study that this protein is not only expressed in disturbed epidermis but also in other epithelia that are constantly exposed to environmental stress and where the presence of inflammatory cells and hyperproliferation is physiological [294]. In general SKALP expression seems to be induced upon hazardous/stressful stimuli in epithelia. As mentioned earlier in this paper the expression of SKALP in the epidermis is correlated with the activation of response proteins like p38. Moreover in cultured keratinocytes FCS and TNF-α inducible SKALP expression is mediated through p38 dependent processes. There is however little known about the regulation of SKALP gene expression at the molecular level. The co-expression of CK6, CK16, CK17 and SKALP either in physiological or pathological conditions suggests common regulatory mechanisms. In this paper we have described the results of experiments intended to reveal the regulatory mechanisms that are directly involved in the regulation of SKALP gene activity and could be indicative for regulation of abnormal epidermal gene expression as well.

By determining the transcription start site of the SKALP gene, the putative SKALP promoter could be positively identified as such. However transcription starts in a different region than was expected, based upon previously designated consensus sequences, resulting in a messenger RNA with a unusually short 5'-untranslated region (5'-UTR) [411]. Originally a sequence ranging from position -94 until -89 (relative to translation start) was thought to function as TATA box [253,256] and transcription was expected to start somewhere around position -60. Our results indicate that transcription can start in a region ranging from 9 to 24 bases upstream of the translation startsite. In view of these results we claim that the TATA-box like sequence at positions -94/-89 is non-functional. At positions

-48/-42 we found a sequence TAAAGAT, that is, with respect to spacing, more likely to serve as the actual TATA box. The fact that this proposed TATA-box deviates from the consensus sequence for TATA boxes (TATA[A/T]A[A/T]) might explain the existence of multiple transcription start points [412]. In a previous paper [299] we have speculated on the regulatory properties of an open reading frame (ORF) in the 5'-UTR (ranging from pos. -37 to -5) of the SKALP messenger RNA. These kind of small ORFs are, once expressed, known to have regulatory properties with respect to translation of the messenger [413-415]. Based upon the results presented in this paper this in no longer a valid option. Transcripts starting between -24-and -12 (instead of around -60) no longer carry this complete ORF in their 5'-UTR.

With respect to transcription factors that might be involved in the initiation of abnormal epidermal gene expression, the CK6 and CK16 genes have been investigated at the molecular level. Several groups have used gel retardation assays to identify a great number of functional transcription factor binding sites in their promoters. These factors include AP1, AP2 and Sp1 [13,45,195,416], suggesting involvement of these transcription factors in regulating the expression of these genes in the context of abnormal differentiation and inflammation. However all these transcription factors have been implicated in the regulation of normal epidermal differentiation as well [325,326]. Nevertheless with respect to AP-1 binding sites, immunohistochemical data have shown that each member of the jun/fos family has its own pattern of expression with respect to the distribution over the *strata*, probably resulting in the presence of specific AP1 complexes in each *stratum* [329,417]. There have been a number of reports that have described the binding of different AP-1 complexes to different AP-1 sites within one promoter that can have both enhancing or repressing effects [418-420]. It might very well be possible that genes expressed during normal differentiation and genes expressed during regenerative maturation are both regulated through different members of the AP1 transcription factor family. In this respect it is also interesting to look at a study in which SKALP promoter activity has been studied in normal mammary epithelial cell [301,330], and which suggests that that SKALP gene expression is differentially regulated between these cells and epidermal keratinocytes. Deletion and mutation experiments showed that an AP1 consensus-binding site at position 440 in the SKALP promoter was important for SKALP expression in these mammary cells. However, in our in vitro model system for the induction of regenerative maturation we did not see any changes in the binding capacities of nuclear proteins to AP-1 consensus sites using EMSA, but this does not rule out a change in AP-1 protein repertoire.

Transfection experiments with SKALP promoter constructs in keratinocytes showed a discrepancy between the expression of these clones and the endogenous gene with respect to inducibility. Whereas the endogenous SKALP gene can be induced in cultured keratinocytes (addition of 5 % FCS), the reporter gene constructs are active under all culture conditions. Analysis of these constructs in keratinocytes under SKALP expressing culture conditions showed a major decrease of promoter activity upon the deletion of a sequence region (-440/-290) containing three NF-IL6 consensus binding sequences. However the EMSA experiments in which an oligo harbouring a NF-IL6 consensus binding sequence was used, did not show any changes in NF-IL6 binding capacities in nuclear extracts from

keratinocytes that (are induced to) express SKALP. As mentioned before identical EMSA results were obtained with an oligo containing and AP-1 consensus-binding site. These experiments suggest that AP-1 and NF-IL6 could still be relevant for SKALP transcription but certainly not sufficient.

Further reduction of the SKALP promoter region down to -130 left only 10 percent of the initial promoter activity. In this region of the SKALP promoter a consensus sequence for the transcription factor NFκB is present. In total the SKALP promoter harbours two NFκB consensus-binding sequences. One located at position -163/-154, fitting the consensus sequence 'GGGRNNYYCC' [169] and one located at position -972/-965, fitting the consensus sequence 'GGGRNTYYC' [421]. The EMSA experiments revealed a rapid activation and translocation of the transcription factor NFκB into the nuclei of keratinocytes that were induced to express SKALP suggesting a possible causal relation. In addition we could confirm this translocation by immunocytochemical analysis of cultured keratinocytes and in a previous paper we have also described the correlation between NFκB translocation and SKALP expression through UV-B irradiation of normal human epidermis (Pfundt, *et al*, submitted). In a study by Ma *et al*, NFκB (p65 homodimer) is also implicated in the possible regulation of CK6 and a synergistical effect for AP-1 and NFκB is suggested [46]. Further mutation analysis is required to analyse the functional role for NFκB is the regulation of SKALP expression. However all together these data suggest a regulatory role for NFκB in the induction of regenerative maturation related gene expression. With respect to consensus binding sequences, analysis of the known 5'-promoter regions of CK6 (381 bp), CK16 (507 bp) and CK17 (450 bp) revealed that only CK17 contains a NFκB consensus binding sequence. This in contrast with the fact that in the study of Ma *et al*, that was mentioned earlier CK17 expression was not affected by the presence of active NFκB. However in this study only the p65 (RelA/RelA) homodimer was used whereas active NFκB complexes can be formed by homo- and hetero- dimerization of a variety of NFκB family member's [169].

The discrepancy between transcription of the naked DNA of promoter constructs and the nuclear DNA wrapped in highly organised chromatin structure has been observed frequently [184,422,423]. Therefore, also other processes like silencing elements not present in the transfection clones, mRNA stability or higher order chromatin structure might be involved in transcription regulation of the endogenous SKALP gene. In that respect p38 kinase activity, which is crucial for SKALP expression *in vitro*, could play a role. Recently it has been described in a number of papers that mRNA's can be stabilised through a MAP kinase mediated processes including p38 [424-429], upon cellular stress. With respect to mRNA stability the somewhat aberrant length of the 5'-untranslated region of the SKALP mRNA could be of some significance, although it has been described that the 3'-UTR is more important for mRNA stability [430-432]. In addition it has also been described in a large number of cell types that (TNF-α mediated) NFκB activation and translocation is regulated by activated MAP kinases and particularly by p38 MAP kinases [433-438]. Based upon the integration of the results presented in this paper into the literature we hypothesise that SKALP expression is part of an epidermal stress response in which p38 and NFκB play a central role and might be functionally linked to each other in activated keratinocytes.

Manipulation of epidermal differentiation *in vivo*

Characterisation of two whole-skin models for studying the disturbance of epidermal homeostasis

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Abstract

The availability of reliable models for epidermal growth and differentiation is crucial for dermatological research. Due to the effort of many research groups, there now are a number of well-established cell culture models in which keratinocyte biology can be studied. These models obviously differ in various respects from the actual situation in skin. It is therefore very important to also develop and characterise model systems that, although more complicated to handle and less flexible, better mimic the *in vivo* situation. Here we present the further characterisation of two whole-skin models in which epidermal growth and differentiation can be studied. The first model is an explant model that uses 4 mm punch biopsies of healthy human skin, which are cultured for 24 hours in serum-containing medium. This culture period itself induces an altered differentiation route in the epidermis of the explants. Based upon *de novo* expression of SKALP/elafin and cytokeratin 16 and the upregulation of SLPI and involucrin we conclude that the differentiation pathway that is induced in these skin explants resembles the differentiation pathway that is found in activated keratinocytes, the so-called regenerative maturation phenotype. The second model that we have characterised is a transplantation model in which normal healthy human skin was transplanted on the back of athymic mice. We observed that after a healing/normalisation period of approximately 40 days the differentiation and proliferation status in the skin grafts resembles normal skin. Based upon the expression profiles of a large number of epidermal markers we concluded that, as in the *in vivo* situation, the regenerative maturation pathway could be induced in these grafts by either tape stripping or UV-B irradiation. In addition the recruitment of a number of stress response proteins that are thought to be involved in the regulation of epidermal responses preceded the transition from normal to abnormal epidermal differentiation.

Introduction

The epidermis is a dynamic, continually renewing tissue that forms the interface between the organism and its environment. The major epidermal cell type, the keratinocyte, undergoes a complex and highly controlled program of terminal differentiation leading to the formation of the horny layer of skin [259,260]. There are, however, conditions in which the keratinocyte deviates from the normal differentiation program and follows an alternative maturation route, called regenerative maturation [6,7]. This regenerative maturation pathway is characterised by abnormal keratinocyte differentiation in the context of epidermal hyperproliferation. This epidermal phenotype is a physiological epidermal adaptation to conditions that disturb the barrier function of the skin and disrupt epidermal homeostasis, like e.g. wounding and UV-B irradiation. There are however situations in which the epidermal phenotype of regenerative maturation does not seem to be of any physiological relevance and, moreover, is the cause of pathological skin conditions in for instance the skin disease psoriasis [10]. Several research groups around the world are trying to get insight into the cellular mechanisms that are at the basis of this switch in epidermal differentia-

tion. Elucidation of these pathways will lead to a better understanding of the regulation of epidermal growth and differentiation and potentially leads to new therapeutic targets in skin diseases where abnormal differentiation is involved.

For scientists, the accessibility of reliable and controllable models in which epidermal phenotypes can be studied is indispensable. Much effort has been put into the development of such models. After the introduction of the feeder cultures by Rheinwald and Green in 1975 [224] most of the experimental work has been performed using primary keratinocytes. The development of media in which keratinocytes can be grown in the absence of serum was a significant advance in controlling the experimental conditions of keratinocyte culture [77,439]. The present culture models of primary keratinocytes are very flexible and large amounts of cells can be obtained for large scale screening experiments. However inherently these in vitro culture models differ considerably from the actual situation in skin, both from a biological and a physical point of view. The fact that these keratinocyte cultures are mainly monocultures means that the influence that other skin cells have in the epidermis is not accounted for in these culture models. In addition the absence of a cornified layer makes it very difficult predict potential irritancy and/or toxicity of compounds *in vivo* because skin penetration may be very poor in intact human skin. And finally, due to the lack of circulation *in vitro*, the clearance and half-life of compounds in culture models is bound to be different in human skin.

The aim of the study described in this paper was to characterise a model in which the switch from normal epidermal differentiation to regenerative maturation in intact human skin could be induced and studied. Furthermore this model should be suitable for testing new active pharmacological compounds or other agents that could potentially interfere with this epidermal differentiation switch.

Here we have used two model systems that have been described previously [236,440,441] but have remained poorly characterised with respect to stress-induced epidermal changes. First we characterise an explant model in which skin biopsies are cultured for 24 hours, and second, a skin grafting model using nude mice is described. Based upon the expression patterns and levels of a number of markers we conclude that regenerative maturation can be studied in these models. In the explant model the regenerative phenotype is induced by the explant procedure itself within 24 hours, whereas in the transplantation model we can induce the abnormal epidermal phenotype by either irradiation of the transplant with UV-B or by tape stripping. These models should, together with *in vitro* models of cultured keratinocytes, provide us with an array of models each with its own pros and cons from which the experimental information can be combined.

Materials and methods

Air-exposed skin explant model

Punch biopsies (4mm diameter) were taken from residual human skin after abdominoplasty. Informed consent was obtained from patients participating in this study. These explants were washed in PBS, and placed with epidermis up in a Transwell insert system

with tissue culture treated polycarbonate membranes (Corning Costar Corporation, Badhoevedorp, the Netherlands). The inserts with the skin explants were put in 24-well tissue culture plates in keratinocyte growth medium (KGM) supplemented with 5% foetal calf serum (FCS) (Seralab, Nistelrode, the Netherlands). KGM was composed of keratinocyte basal medium (KBM) ((Clonetics, San Diego, CA, USA); 0.15 mM calcium) supplemented with ethanolamine (0.1 mM, Sigma, St. Louis, MO, USA), phosphoethanolamine (0.1 mM, Sigma), bovine pituitary extract (BPE) (0.4%, $^{v}/_{v}$, Clonetics), recombinant mouse epidermal growth factor (EGF) (10 ng/ml, Sigma), insulin (5 µg/ml, Sigma), hydrocortisone (0.5 g/ml, Collaborative Research Inc. Lexington, MA, USA), and penicillin plus streptomycin (Life Technologies, USA). The *stratum corneum* of the explant was air-exposed. The explants were incubated for 24 h at 37˚C under a 92% humidified atmosphere containing 5% CO_2 in air. Subsequently, they were further prepared for immunohistochemical examination.

Mice

Female athymic nude BALB/C mice of 6 to 8 weeks were used as recipients for human skin grafts. All mice were housed in filter-top cages, away from other animals, in specific barrier-sustained conditions to decrease the incidence of infections. They were maintained on autoclaved feed and acidified-chlorinated water, and bedding that was sterilised and changed weekly.

Grafting procedure

Informed consent was obtained from skin graft donors participating in this study. A human skin sample of 0.6-mm thickness was taken with a keratotome from residual normal human skin after abdominoplasty. Samples were prepared for grafting by cutting into circular pieces with 8-mm biopsy forceps. The mice were anaesthetised with ether and a full thickness bed of 8 mm in diameter was prepared for the grafts. Each mouse received two skin grafts on the upper-thoracic region, one on either side of the midline on the dorsal surface of the mouse. Grafts were fitted carefully into the graft bed, and the area was bandaged with a sticking plaster and self-adhesive tape. On the 10th day after grafting, the adhesive tape and plaster were removed carefully and the grafts were then inspected at regular time intervals. After the stated periods of time, up to 72 days after transplantation, the mice were sacrificed and the grafts were removed in total with a small strip of surrounding mouse skin. Tissues were further prepared for immunohistochemical examination.

UV irradiation of human skin grafts

Human skin grafts on the back of the mice were irradiated with UV-B. Therefore, the mice were anaesthetised by i.p. injection of 200 mg/kg ketamine (Nimatek®, Eurovet, Bladel, the Netherlands). To be sure that only the human skin grafts would be irradiated, the mice were wrapped in aluminium foil with an 8-mm gap. Ultraviolet light was generated using a Fluo-Link (Vilber Lourmat) with a peak wavelength of 311 nm. One of the two grafts on each mouse was irradiated with either 1.0 or 1.5 J/cm^2 UV; the remaining graft was used

as negative control. Body temperature was monitored and kept constant by the use of a warming blanket and lamp. Mice were sacrificed either 2 h or 48 h after UV-B irradiation and the skin grafts were removed and prepared for immunohistochemical examination.

Immunohistochemistry

Skin specimens were fixed for 4 h in buffered 3.8% formalin and processed for embedding in paraffin. Care was taken to embed the specimens so that sections could be cut perpendicular to the skin surface. Sections (6 μm) were mounted on 3-aminopropyltriethoxysilane (Sigma) coated slides and were subsequently deparaffinised and rehydrated. For antigen retrieval from paraffin sections, the slides were pre-treated two times 5 min in 10 mM citrate buffer for LL025 (CK16), for MIB-1 (Ki-67) and for Mon150 (involucrin) a pre-treatment of 10 min in 10 mM citrate buffer using a microwave oven (Miele, M720) at 450 W was performed. Sections to be stained for CK10 were pre-treated with trypsin for 15 min. For immunohistochemical detection of SKALP, SLPI and NFκB no pre-treatment was necessary. After pre-incubation with 20% normal swine, goat, or horse serum (Vector Laboratories Inc., Burlingame, CA) the slides were incubated for 60 min with the primary antibodies and washed with PBS. Samples for the detection of SLPI, CK16, involucrin, CK10, and Ki-67 were subsequently incubated with the appropriate biotinylated secondary antibody for 30 min. To enhance the staining, the sections were incubated with Avidin-Biotin-Complex (ABC) after which they were developed with metal enhanced DAB or aminoethylcarbazole (AEC) (Sigma) as a chromogenic substrate. Samples for the detection of SKALP were incubated with peroxidase-conjugated swine-anti-rabbit Ig or (Vector Laboratories Inc.). Apoptotic cells were identified using TUNEL staining as described before [357]. Immunohistochemical detection of phosphorylated p38 was performed using an enhancement kit for rabbit polyclonal antibodies (CSA kit, DAKO). If necessary, the slides were counterstained with Mayer's haematoxylin (Sigma) and mounted in glycerol gelatine. Appropriate controls with pre-immune sera, blocking peptides or omission of the primary antibodies were performed.

Antisera

A polyclonal antiserum against recombinant SKALP was obtained as described previously [309]. An antiserum against a synthetic peptide comprising amino acids 23 to 36 of SKALP was prepared by coupling the peptide via a C-terminal cysteine residue to chicken ovalbumin, using the sulfo-SMCC procedure according to the manufacturer's instructions (Pierce, Rockford, IL, USA). This conjugate was used for immunisation of a rabbit according to previously described protocols [238]. Control serum (pre-immune serum) was drawn before the immunisation procedure. The antiserum against the synthetic peptide was affinity purified using the synthetic peptide coupled to Sulfolink coupling gel according to the manufacturer's instructions (Pierce). The two antisera against recombinant SKALP and against the synthetic peptide gave identical staining patterns in histological sections. Antisera against SLPI were prepared, characterised, and used as described before [356,442]. In this study we used a monoclonal serum (clone 31) against SLPI, which shows no cross-reactivity with SKALP. Mon150, a monoclonal antibody against involucrin was obtained

as previously described [263], and LL025, a monoclonal antibody recognising CK16 was from Novocastra. For detection of Ki-67, the MIB-1 antibody of Immunotech was used, and RKSE60 was used to detect CK10. Dually phosphorylated p38 was detected by using a rabbit polyclonal antibody from New-England Biolabs. And finally the detection of NFκB was performed with a rabbit polyclonal antibody from Santa-Cruz directed against the p65 subunit of the NFκB transcription factor complex.

Results and discussion

Human skin models have proven their usefulness, since studies on humans are often considered ethically unacceptable, very time-consuming when performed on a large scale basis, and difficult to interpret due to large inter-individual variability. Ideally, skin models should approach the source tissue very closely and have a high predictive potential for the in vivo situation. In the past several years three-dimensional systems have been developed that are based on the recombination of cultured cells in order to form a reconstructed epidermis which approaches the in vivo situation much more closely then in vitro monolayer cultures. Nowadays, growing differentiated keratinocyte cultures on cell-free or fibroblast-populated dermal substrates can create various types of skin systems. Examples of substrates are dead de-epidermized dermis [443], inert filter substrates [234], and fibroblast-populated collagen matrices [444]. Morphological studies have shown that these skin equivalents form a multilayered epithelium and express markers of epidermal differentiation [445]. These three-dimensional skin equivalents are however not optimal for all studies, because they only consist of two cell types (keratinocytes and fibroblasts). Especially when studying inflammation or other immunological processes, other cell types such as melanocytes, Langerhans cells, and Merkel cells, may play important key roles. So, despite the fact that the use of these three-dimensional skin equivalent systems in vitro has resulted in a considerable progress in the understanding of keratinocyte biology, other organotypic systems and mouse models have additional potential advantages for scientific research. The aim of the study described in this paper was to develop and characterise methods by which the switch from normal epidermal differentiation to regenerative maturation could be induced and studied in intact skin. In this report we use two skin models that have been described previously by other groups: an organotypic air-exposed skin explant model [236,440] and a grafting mouse model [441]. These two models were analysed immunohistochemically and compared with each other with respect to their response in differentiation and proliferation.

Air-exposed skin explant model

The first model that was characterised in this study is the air-exposed skin explant model. In this model we use 4 mm punch biopsies of intact normal human skin that are maintained in tissue culture inserts in KGM supplemented with 5 % FCS for 24 hours. During this period, the *stratum corneum* was air-exposed. We evaluated expression levels and patterns of a number of genes that are regularly used as markers for epidermal proliferation (Ki-

67), normal differentiation (CK10), and abnormal regenerative maturation (SKALP, SLPI, CK16, involucrin). The markers for regenerative maturation are known to be strongly induced (SKALP, CK16) or upregulated (SLPI, involucrin) in response to the disruption of epidermal integrity like injured skin and during skin inflammation, as found in the skin disease psoriasis.

Figure 1 shows immunohistochemical staining of the different markers in our explant model. In explants cultured for 24 h, SKALP and SLPI (two proteinase inhibitors) were strongly expressed in the granular layers. Some keratinocytes of the spinous layers also tend to express SLPI. CK16 showed a minor increase in expression level (mainly in the *stratum granulosum*), whereas involucrin was grossly upregulated in comparison to normal skin. Involucrin was present in spinous and granular layers of cultured explants. This expression pattern was similar to that of submerged keratinocyte cultures, in which expression of SKALP, SLPI, CK16, and involucrin can be induced/upregulated by the addition of 5% FCS. [77,356]. The 24 h-incubation period did not alter the expression levels of CK10 and Ki-67. CK 10 was localised in the suprabasal layers and several basal keratinocytes showed expression of Ki-67, this is similar to their expression patterns in normal epidermis. Based upon these expression patterns we hypothesise that regenerative maturation can be induced through a 24 h-incubation of normal human skin explants in culture. In principle this makes sense, as during this 24-h of incubation the keratinocytes in the margins of the explant react by inducing the process of wound healing, which is known to be attended by the initiation of regenerative maturation.

This explant model could be useful to examine factors involved in regenerative maturation or wound healing processes. In contrast to submerged culture models, tissue architecture is still similar to that of native skin, all epidermal strata are present with comparable appearance. There are however also limitations of this explant model, for example the lack of a vasculature and of migrating inflammatory cells. The ultimate manifestation of inflammation can not be simulated in this system. Furthermore, the lifespan of explants in culture covers a maximum of 24 h and therefore long-term studies are limited.

Skin grafting mouse model

Although laws and regulations are becoming more stringent regarding laboratory tests on animals, animal models are still valuable tools in scientific research. We have used a model of human skin grafted onto nude mice and investigated if we could switch the epidermal differentiation pattern from normal to regenerative. For examination of the differentiation pattern of the keratinocytes, immunohistochemical staining was performed using antibodies directed against the cytokeratins CK10 and CK16 (specific keratinocyte differentiation markers), SKALP and SLPI (proteinase inhibitors and antimicrobial proteins), involucrin (precursor of the cornified envelope), and Ki-67 (proliferation marker). Initially the expression pattern of the various marker genes was investigated at different time-points during a period of 72 days after transplantation. As shown in figure 2, at day 15 (the first time-point) after transplantation the skin grafts show expression of the proteinase inhibitors SKALP and SLPI, which is restricted to the keratinocytes in the granular layer. In addition the expression level of CK16 is very high in all suprabasal layers of

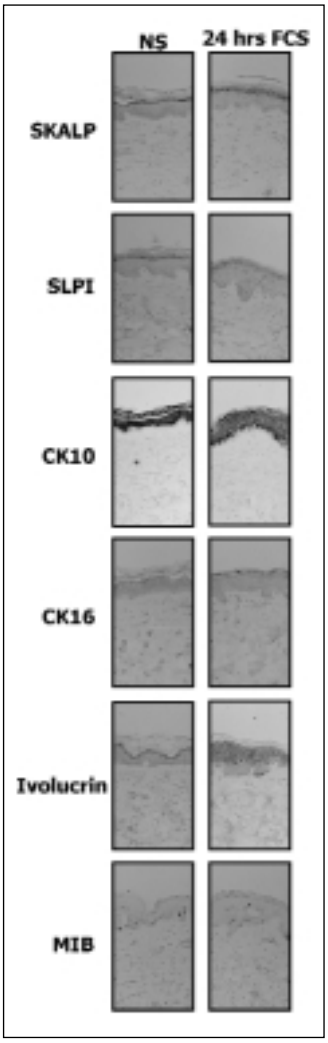

Figure 1. Immunohistochemical analysis of differentiation and proliferation related gene expression in the air-exposed skin explant model. Expression patterns of SKALP, SLPI, CK10, CK16, involucrin, and Ki-67 (MIB) in skin explants that were cultured in KGM supplemented with 5 % FCS (second column) were compared to those in normal skin (NS (first column)). The culture period of 24 hours results in a slight increase in the expression of genes associated with abnormal epidermal differentiation e.g. SKALP, SLPI, CK16. The expression of involucrin is also markedly increased whereas the proliferation rates in the basal layers are unaffected by the culturing period.

the transplant, whereas involucrin is only highly expressed in upper spinous and granular layers. Furthermore, the transplants show an elevated proliferation rate, since the number of keratinocytes in the basal layer that stain positively for the Ki-67 antibody (MIB) is increased. CK 10, a cytokeratin that is generally used as a marker for normal keratinocyte differentiation, is expressed in all suprabasal layers, and its expression level remain unchanged during the whole period of 72 days (not shown). Moreover we also observed a thickening of the *stratum corneum* of the skin following transplantation (see figure 2). Acanthosis of human skin grafts was found earlier by Bruengger et al. [446], who presumed an adaptation to the changed mechanical stress. In summary, expression levels of proteins that are absent or only slightly expressed in normal human epidermis but associated with

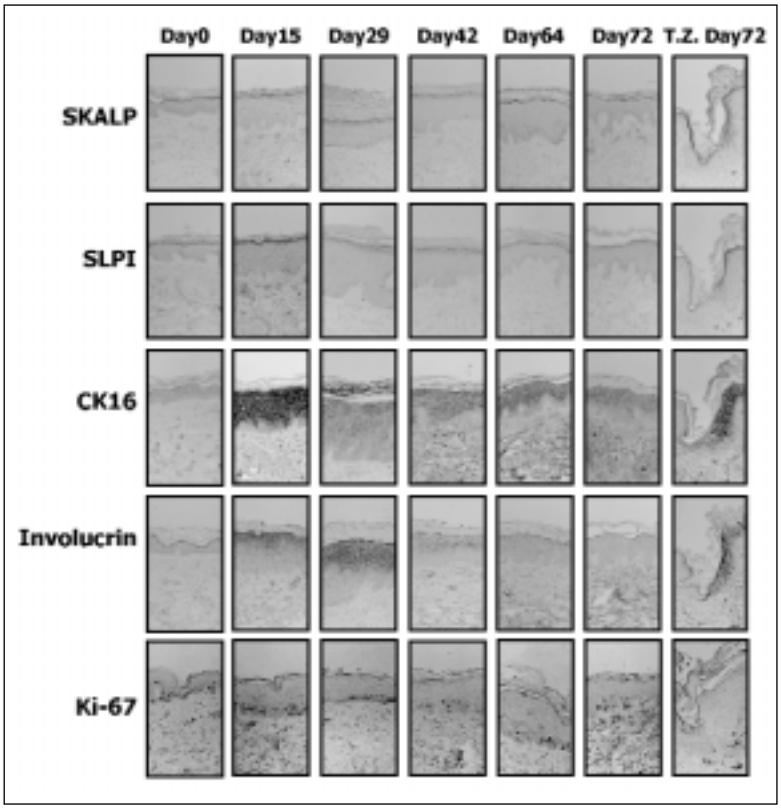

Figure 2. Immunohistochemical analysis of differentiation and proliferation related gene expression in the skin grafting model on nude mice. Expression patterns of SKALP, SLPI, CK16, involucrin, and Ki-67 (MIB) were analysed at day 15 (column 2), 29 (column 3), 42 (column 4), 64 (column 5) and 72 (column 6) after the transplantation of human skin and compared to normal skin (first column). In the last column the transition zone between recipient mouse skin and transplanted human skin is shown. The expression of SKALP, SLPI, CK16, and involucrin is increased in freshly transplanted skin until at least day 42. The apparent positive staining of SKALP at day 0 is caused by counterstaining. At timepoints 15, 29 and 42 days after transplantation, a slight increase in SKALP expression in the granular layer and the first layers of the spinous layer was found. Normal skin stains positive for SLPI expression in the granular layer. At day 15, 29, and 42 SLPI is also expressed in the upper layers of the stratum spinosum after which the expression returns to normal levels. CK16 expression is absent in normal skin and found to be induced in the upper spinous layers at day 15, 29, and 42 after which CK16 expression normalises. The use of a monoclonal antibody against CK16 results in rather high background staining (especially found at day 42 and later). Involucrin is expressed in the stratum granulosom in normal skin. In freshly transplanted mice (day 15 and 29) involucrin expression is found throughout the stratum spinosum as well. At day 64 the expression of involucrin returns to normal patterns. The proliferation rate of the basal keratinocytes is slightly increased in transplanted human skin until at least day 29 (nuclei that stain positive for Ki-67 in the basal layers). After day 42 the number of proliferative basal cells normalise to initial levels. The spinous layers of the skin transplants at the transition zone between mouse skin and human skin (last column) remain positive for SKALP, SLPI, CK16 and involucrin. The proliferation rate of the basal keratinocytes in this region is also increased compared to normal human skin (day 0) as witnessed by positive staining for Ki-67 in the basal nuclei.

wound repair, hyperproliferation, and abnormal differentiation, are upregulated at day 15 after transplantation. In course of time, expression patterns of these markers that were initially upregulated, normalise to basal levels comparable to levels in normal human skin. The transition zone between mouse skin and human skin transplant forms an exception. These transplant margins remain positive until at least day 72 for SKALP, SLPI, CK16, involucrin, and Ki-67 expression. So, except for the transplant margins, at day 72 after transplantation, human skin transplants show an expression pattern of proliferation and differentiation-specific genes that is essentially similar to normal differentiation.

Having established that already 30-40 days after transplantation the grafted human skin mimics normal human skin, a method was sought to switch the epidermal differentiation from normal to regenerative. It is known that regenerative differentiation is induced in situations of epidermal stress. We therefore examined the effects of two stress-inducing conditions, ultraviolet (UV)-B irradiation and tape stripping, on expression levels of the investigated genes. Recently it was shown that UV-B irradiation of keratinocytes in vitro and human skin in vivo leads to the activation of cellular stress response pathways, which is exhibited by activation of p38, JNK, NFκB, AP-1, and to a lesser extent ERK. [130,133,339]. Examples of UV-B induced alterations in gene expression levels of human keratinocytes are the upregulation of ICAM-1 [348], upregulation of CK 1/10 and 5/14 [349], and induction of CK 16 [350]. Very recently, we found that SKALP, SLPI, CK16 and involucrin expression was induced suprabasally after irradiating human skin with 2 MED of UV-B light. This induction of gene expression was preceded by activation of cellular stress response pathways such as phosphorylation of c-jun and p38 MAP kinase (Pfundt et al. submitted). Another stress-inducing condition that is known to cause increased SKALP and SLPI expression in vivo is tape stripping. Through the repeated application and removal of adhesive tape the *stratum corneum* is removed. This procedure can be used as a model for standardised epidermal injury of the epidermis and epidermal osmotic stress [14,202,356]. We were interested to know whether we could induce regenerative maturation by either UV-B irradiation or tape stripping in human skin transplants. In figure 3, expression levels of the earlier mentioned protein markers were analysed after either irradiation with 1.5 J/cm^2 UV-B or tape stripping and compared with expression levels in untreated skin transplants. Expression of SKALP, SLPI, CK16 and involucrin is induced, both 48 hours after irradiation and 48 hours after tape tripping indicating the induction of abnormal epidermal differentiation. The localisation pattern of this protein expression is however different between UV-B irradiation and tape stripping. After irradiation, the upper spinous layers and the granular layers of the epidermis stain negative for all the markers. A band of positive cells can be found in the middle layers of the *stratum spinosum*. Based on the morphology of the upper spinous and the granular layers and the presence of sunburn cells in these locations we conclude that these cell layers are heavily damaged by the irradiation procedure and are/will become apoptotic (see figure 3). The presence of apoptotic cells in these cell layers is described below. The middle cell layers that have received lower doses of UV-B radiation respond by altering their gene expression patterns. This in contrast to the tape stripping procedure which is obviously less damaging. Forty-eight hours after the stripping procedure expression of SKALP, SLPI, CK16 and involucrin can

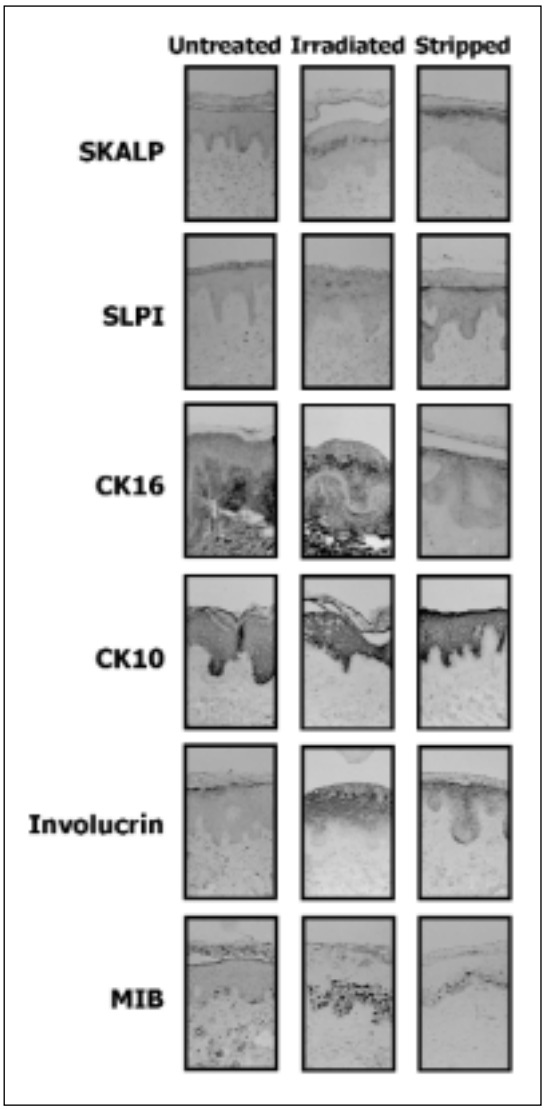

Figure 3. Immunohistochemical analysis of differentiation-related gene expression in the grafting mouse model 48 hours after UV-B irradiation (second column (1.5 J/cm^2)) and tape stripping (third column) compared to untreated skin graft (first column). The expression levels of SKALP, SLPI, CK16, CK10, involucrin, and Ki-67 (MIB) are shown. Irradiation of the skin grafts results in induction of SKALP, SLPI, CK16 and involucrin gene expression in the middle layers of the *stratum spinosum*, and massive induction of cell proliferation in the basal layer(s). After tape stripping SKALP, SLPI, CK16 and involucrin expression is also induced but in the upper layers on the *stratum spinosum* and the *stratum granulosum*. In addition tape stripping also results in high proliferation rates in the basal layers(s). Expression of cytokeratin 10 (CK10) remains unchanged.

be found in the upper spinous and granular layers (see figure 3). Expression levels of CK10 remain constant, this was also found during 72 days after transplantation. Furthermore, irradiation and tape stripping lead to an elevated proliferation rate, since massive numbers of keratinocytes in the basal layer stain positive with the Ki-67 antibody (MIB). This indicated that epidermal hyperproliferation is induced both after UV-B irradiation and tape stripping.

It is known that the effect of UV-B irradiation of human skin is dose-dependent. High doses can lead to the formation of apoptotic (sunburn) cells [351,352], whereas lower doses of

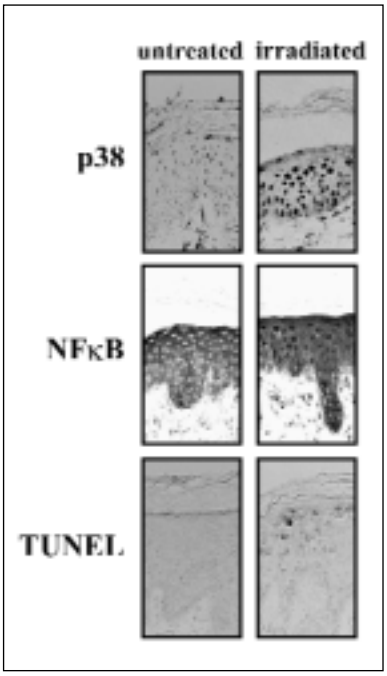

Figure 4. Analysis of the cellular stress related proteins p38 (row 1) and NFκB (row 2), 2 hours after UV-B irradiation (second column) compared to untreated skin graft (first column). Phosphorylated p38 is absent in untreated skin grafts, but in response to UV-B irradiation high levels of dually phosphorylated p38 were found throughout the epidermis (row 1, column 2). The transcription factor NFκB is localised cytoplasmic in untreated skin grafts and translocated to the nuclei within 2 hours after UV-B irradiation (row 2, column 2). Forty-eight hours after UV-B irradiation the presence of apoptotic cells was analysed using TUNEL staining. In untreated skin grafts no TUNEL positive cells were detected (first column, row 3) whereas after UV-B irradiation apoptotic keratinocytes could be detected in the upper layers of the *stratum spinosum* and *stratum granulosum* (second column, row 3).

UV-B allow cell survival and repair of genetic damage [354]. In the upper epidermal layers of all irradiated transplants we observed hotspots of keratinocytes with apoptosis resembling features (cell shrinkage and fragmentation). Therefore we performed TUNEL assays, which show that these hotspots indeed contain apoptotic keratinocytes (figure 4). Based on earlier studies (Pfundt et al., submitted) we examined the presence and/or activation patterns of two proteins involved in stress-induced signal transduction pathways i.e. p38 and NFκB, following UV-B irradiation of the human skin transplants. As shown by immunohistochemical staining (figure 4) both proteins are affected by the irradiation procedure. In the keratinocytes of the grafted human skin NFκB is localised mainly cytoplasmic, whereas 2 hours after UV-B irradiation NFκB is translocated from the cytoplasm to the nucleus of the keratinocytes (see figure 4). Phosphorylated (hence activated) p38 is present in untreated skin grafts in very small amounts (see figure 4). However after irradiation the skin graft within 2 hours massive amounts of active p38 are present in the nuclei of keratinocytes throughout the epidermis. This apparent recruitment of stress related proteins to the nuclei of irradiated keratinocytes suggest involvement of these proteins in nuclear processes like DNA repair programs or transcriptional activities. It should however be noted that the dose of UV-B that was used in this experiment is considerably higher than doses that are generally used in studies with normal human skin. In earlier performed studies using healthy human volunteers we have found identical results, with respect to protein expression patterns and stress-protein recruitment, using approximately a four-fold lower UV-B dose. We suspect that the enhanced thickening of the *stratum*

corneum in the skin grafts that was mentioned earlier could very well cause this difference. This hypothesis is confirmed by the observation that the activation of p38 and NFκB was clustered on zones where the *stratum corneum* was obviously thinner (not shown).

In conclusion, the explant model and the transplantation model have great potential value for studies aimed at the elucidation of the factors involved in the switch in epidermal differentiation. These models are attractive because different cell types and environmental factors leading to regenerative differentiation may be studied in human tissue. In the explant model, regenerative differentiation is induced within 24 h by the explant culture procedure itself, whereas in the skin grafting mouse model, UV-B irradiation and tape stripping can induce this abnormal epidermal phenotype. Furthermore these models may be suitable for testing new active pharmacological compounds or other agents that could potentially interfere with this epidermal differentiation switch. However, investigators should keep in mind that the grafted mouse model is still a 'two-species-system' and the preparation time can run to several months. In contrast, the air-exposed skin explant system is easy to handle, but long-term studies are restricted to 24 h due to the limited lifespan of the explants. Overall, although there is no model imitating the in vivo situation completely, many aspects of normal and regenerative maturation are mirrored in the currently available models, including in vitro submerged keratinocyte cultures, three-dimensional skin equivalent systems, organotypic explant model, and grafting mouse model.

Sequence-specific inhibition of gene expression in intact human skin by epicutaneous application of chimeric antisense oligodeoxynucleotides.

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Abstract

Targeted and selective inhibition of keratinocyte gene expression in human epidermis could be an efficient and safe pharmacological approach in a number of skin diseases. In this study we investigated if topical application of antisense oligodeoxynucleotides (ODNs) on intact human skin, can be used to inhibit expression of a gene in the differentiated compartment of the epidermis. We applied a variety of 20-mer antisense and control ODNs designed to hybridise to different regions on the mRNA of the inducible epidermal proteinase inhibitor SKALP/elafin that was used as a model target gene. When nuclease-resistant fully phosphorothioate ODNs were applied to explant cultures of human skin, they were found to be either ineffective at low doses or severely toxic at higher doses which could be due to the extremely high degree of protein binding found with this type of ODNs. When chimeric ODNs with a phosphodiester core and phosphorothioate 5' and 3' ends were applied to intact skin no toxicity was noted. One of the tested chimeric ODNs, that exhibit only minor protein binding, was found to inhibit SKALP expression at the protein level in a dose-dependent manner. The observed inhibition on SKALP expression levels was specific as evaluated by application of strict criteria. Sequence-specificity was assessed by the addition of sense and scrambled ODNs that were ineffective. Furthermore, the expression levels of three other differentiation-related genes (involucrin, cytokeratin 16 and SLPI) were not affected, indicating that the inhibition was gene-specific. Confocal laser scanning analysis of fluorescently labeled ODNs confirmed that these molecules can easily penetrate the epidermis and localize in the cytoplasm of differentiated keratinocytes. We conclude that topical application of antisense ODNs can be used to modulate epidermal gene expression, and could potentially be useful to inhibit expression of genes that are relevant in skin diseases.

Introduction

Antisense technology has gained increasing experimental and clinical interest as a tool to modulate expression of a single gene, although considerable skepticism has been justly expressed because of poorly controlled studies and non-sequence specific effects of phosphorothioate oligodeoxynucleotides (ODN) [447,448]. The basis for antisense ODN-based therapies is the formation of an antiparallel duplex by complementary base pairing between the antisense ODN and the targeted mRNA. This results in the inhibition of translation either by cleavage of the mRNA by RNaseH or through steric hindrance of the ribosomal translation apparatus. The highly selective targeting of exact nucleic acid sequences is a great advantage of this technique. The specific inhibition of gene expression is preferred over the working mechanism of conventional drugs that are targeted at whole pathways, complexes, or cell systems. The potential usefulness of antisense ODNs to inhibit gene expression in vivo has been studied by several investigators and it is expected that it will lead to new therapies for a number of human diseases. Nowadays these compounds are already being investigated as therapeutic agents for the treatment of viral infections,

cancers, and inflammatory diseases [449-451]. The most widely used, nuclease-resistant, ODNs are the phosphorothioate (PS) ODNs, where one of the non-bridging oxygens in the phosphodiester backbone is replaced with a sulfur. However, there are serious doubts about the specificity of these compounds, because they were also found to exhibit effects on cells that are not due to antisense mechanisms [448,452,453]. These non-antisense effects of PS ODNs can be either sequence-dependent or sequence-independent. Examples of reported sequence-independent effects are SP1 induction [454], and binding of proteins such as basic fibroblast growth factor, laminin, and fibronectin [455,456]. There are also reports of PS ODN sequences that cause sequence-dependent non-antisense effects. For example, three or more consecutive guanosine residues in a row that form G quartets resulting in the formation of ODN aggregates and inhibition of phospholipase A2 enzyme activity [457]. In addition, unmethylated CpG motifs promote B-cell activation and the production of proinflammatory cytokines, including TNF-α, IFN-γ, IL-6, and IL-12 [458-460]. It has become clear that the antisense technology requires careful experimental design and interpretation of results, since it is complicated by frequent non-sequence dependent effects, and even the control ODNs can exhibit sequence-dependent effects.

Because human skin is very accessible in contrast to most internal organs, diseases involving aberrant gene expression in the epidermis are likely candidates for treatment by antisense ODNs. We therefore started to explore the uptake of ODNs and modulation of gene expression in cultured keratinocytes. Although ODN are readily taken up by keratinocytes [461], in our hands ODNs were not useful to study modulation of gene expression in cultured keratinocytes, since the observed effects on two targeted genes could not be ascribed to sequence specific antisense action (Wingens *et al*, submitted). Subsequent experiments, however, revealed that the uptake and subcellular localisation of ODN was different between cultured cells and intact skin. We therefore reasoned that the failure of finding a specific effect *in vitro* did not preclude the possibility that ODNs could be effective in another experimental system. Therefore, we examined the application of antisense ODNs in an air-exposed skin explant system that resembles the *in vivo* situation. The gene targeted in this study is skin derived antileukoproteinase (SKALP), also known as elafin [293], which we have extensively characterised at the cell biological and genetic level [246,408]. SKALP is, for various reasons, very suitable as a model gene to investigate antisense action. Its expression can be induced in the upper layers of the epidermis by a 24-h incubation in growth medium supplemented with 5% fetal calf serum. In addition, SKALP expression levels can easily be followed on mRNA and protein level. An important control, very often overlooked in antisense studies is gene-specificity of the applied ODN. In the case of SKALP, specific inhibition can be proven by checking the expression levels of the closely related gene coding for secretory leukocyte proteinase inhibitor (SLPI), a homologous proteinase inhibitor with similar expression patterns and regulation mechanisms, as was recently described [356,361]. Expression patterns of unrelated genes either constitutively expressed such as involucrin, or inducible such as cytokeratin 16 (CK16), and the effect of sense and scrambled ODNs were used as additional controls. Our results show that if these strict criteria are applied, target specific inhibition of SKALP gene expression can be demonstrated in this air-exposed skin culture model.

Materials and methods

Air-exposed skin explant model

Punch biopsies (4mm diameter) were taken from residual human skin after abdominoplasty. Informed consent was obtained from patients participating in this study. These explants were washed in PBS, and placed with epidermis up in a Transwell insert system with tissue culture treated polycarbonate membranes (Corning Costar Corporation, Badhoevedorp, the Netherlands). The inserts with the skin explants were put in 24-well tissue culture plates in keratinocyte growth medium (KGM) supplemented with 5% fetal calf serum (FCS) (Seralab, Nistelrode, the Netherlands). KGM was composed of keratinocyte basal medium (KBM) ((Clonetics, San Diego, CA, USA); 0.15 mM calcium) supplemented with ethanolamine (0.1 mM, Sigma, St. Louis, MO, USA), phosphoethanolamine (0.1 mM, Sigma), bovine pituitary extract (BPE) (0.4 % $^{v}/_{v}$, Clonetics), recombinant mouse epidermal growth factor (EGF) (10 ng/ml, Sigma), insulin (5 µg/ml, Sigma), hydrocortisone (0.5 µg/ml, Collaborative Research Inc. Lexington, MA, USA), and penicillin plus streptomycin (Life Technologies, USA). The stratum corneum of the explant was air-exposed, and the ODNs were added locally on top of the skin explants. The explants were incubated at 37˚C under a 92% humidified atmosphere containing 5% CO_2 in air.

Oligodeoxynucleotides

HPLC-purified oligodeoxynucleotides (ODNs) were purchased from Eurogentec S.A. (Seraing, Belgium) and resuspended in sterile water. Two chemically different ODNs were used: the most often used phosphorothioate (PS) ODNs, and chimeric only partially phosphorothioated (PSPO) ODNs. The chimeric ODNs have a phosphodiester core and contain three phosphorothioated linkages at the 5' and 3'ends. All ODNs were 20 nucleotides in length and were designed to optimize for sites with maximal melting temperature (Tm) and minimal self-complementarity. Furthermore, G-quartets and CpG-sequences were avoided as much as possible, since these sequences are thought to cause unwanted site-effects [448,460]. The ODNs were complementary to four different regions of the human SKALP mRNA (figure 1) and the characteristics and sequences of the ODNs are listed in Table 1. The concentration of the ODN was determined by their optical density at 260 nm. From a stock solution of 500 µM, 1 µl (0.5 nmol) or 5 µl (2.5 nmol) was pipetted on top of the skin explants and incubated for 24 h at 37˚C under a 92% humidified atmosphere containing 5% CO_2 in air. After ODN incubation, the protein expression levels and RNA expression levels in the explants were studied by immunohistochemistry and Northern blotting respectively. For the determination of ODN uptake and distribution in the skin explants we used fluorescently labeled ODNs and a Biorad MRC-600 confocal laser-scanning microscope (CLSM) with an argon laser. CLSM allows an optical section to be cut through cells and even through whole explants and is therefore very suitable for the determination of the intracellular localisation of ODNs.

Table 1 Characteristics of SKALP ODNs used in this study.

Name	ODN	target site	sequence (5'-3')	backbone
PSASSK5	antisense	coding region exon 2	GCACTTCTTGATTCCTGGGC	PS
PSSESK5	sense	coding region exon 2	GCCCAGGAATCAAGAAGTGC	PS
PSSCSK5	scrambled	coding region exon 2	GGGTTATGCCTTCCAGCCTT	PS
PSPOASSK5	antisense	coding region exon 2	GCACTTCTTGATTCCTGGGC	PSPO
PSPOSESK5	sense	coding region exon 2	GCCCAGGAATCAAGAAGTGC	PSPO
PSPOSCSK5	scrambled	coding region exon 2	GGGTTATGCCTTCCAGCCTT	PSPO
PSPOASSK6	antisense	translation start site	AAGAAGCTGCTGGCCCTCAT	PSPO
PSPOSESK6	sense	translation start site	ATGAGGGCCAGCAGCTTCTT	PSPO
PSPOSCSK6	scrambled	translation start site	TACACGCGTATGCTAAGCCG	PSPO
PSPOM1SK6	mismatched	translation start site	AAGAATCTGCTGGCCCTTAT	PSPO
PSPOM2SK6	mismatched	translation start site	AAGAAGACATACGCCCTCAT	PSPO
PSPOASSK7	antisense	coding region exon 1	TGAGGAACACCACCACGATC	PSPO
PSPOASSK8	antisense	transglutaminase domain	GTCTTGACCTTTAACAGGAA	PSPO
POASSK5	antisense	coding region exon 2	GCACTTCTTGATTCCTGGGC	PO
POASSK6	antisense	translation start site	AAGAAGCTGCTGGCCCTCAT	PO

All ODNs used are 20 nucleotides in length. Sense control ODNs are complementary to the antisense sequence; scrambled ODNs have the same base composition as the antisense ODNs, though in random order. Underlined nucleotides represent mismatches. PS=phosphorothioate, PO=phosphodiester, PSPO=chimeric phosphodiester ODN with three phosphorothioate linkages at 5' and 3' ends.

Antisera

A polyclonal antiserum against recombinant SKALP was obtained as described previously [255]. An antiserum against a synthetic peptide comprising amino acids 23 to 36 of SKALP was prepared by coupling the peptide via a C-terminal cystein residue to chicken ovalbumin, using the sulfo-SMCC procedure according to the manufacturer's instructions (Pierce, Rockford, IL, USA). This conjugate was used for immunisation of a rabbit according to previously described protocols [238]. Control serum (pre-immune serum) was drawn before the immunisation procedure. The antiserum against the synthetic peptide was affinity purified using the synthetic peptide coupled to Sulfolink coupling gel according to the manufacturer's instructions (Pierce). The two antisera against recombinant SKALP and against the synthetic peptide gave identical staining patterns in histological sections. Antisera against SLPI were prepared, characterised, and used as described before [356,462]. In this study we used a monoclonal serum (clone 31) against SLPI, which shows no cross-reactivity with SKALP. Mon150, a monoclonal antibody against involucrin was obtained as previously described [263], and Ks8.12, a monoclonal antibody recognising CK13 and 16 was from Sigma.

Immunohistochemistry

Explants were fixed for 4 h in buffered 3.8% formalin and processed for embedding in

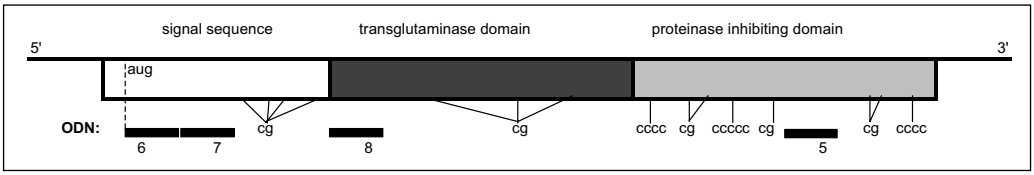

Figure 1. Schematic representation of the organization of the SKALP mRNA and the antisense ODN targets. The mRNA encodes for three protein domains: the signal sequence, the transglutaminase domain, and the proteinase inhibiting domain. The selected antisense ODNs of 20 nucleotides in length were complementary to one region in each domain and another one covered the translation initiation codon *(aug)*. Several segments with frequent occurrence of *cg* or *cccc* sequences were avoided.

paraffin. Sections (6m) were mounted on 3-aminopropyltriethoxy-silane (Sigma) coated slides. Sections were deparaffinised, rehydrated, and used for an indirect peroxidase technique. For antigen retrieval from paraffin sections, the slides were pretreated for 30 min in 10 mM citrate buffer for Ks8.12, and for Mon150 a pretreatment of 10 min in 10 mM citrate buffer using a microwave oven (Miele, M720) at 450 W was performed. After preïncubation with 20% normal swine or rabbit serum (Vector Laboratories Inc., Burlingame, CA) the slides were incubated with the antibodies and after washing with PBS they were incubated with peroxidase-conjugated swine-anti-rabbit Ig or rabbit-anti-mouse Ig (Vector Laboratories Inc.) for 30 min. A solution of aminoethyl carbazole (AEC) (Sigma) in sodium-acetate buffer pH 4.9 containing 0.01% H_2O_2 was added for 15 min after preincubation with sodium-acetate buffer pH 4.9. If necessary, the slides were counterstained with Mayer's haematoxylin (Sigma) and mounted in glycerol gelatin. Appropriate controls with pre-immune sera or omission of the primary antibodies were performed.

RNA-extraction and Northern blot analysis

Total RNA was extracted from explants using RNase-All (2.1 M Guanidine thiocyanate (Research Organics Inc., Cleveland, USA), 8.5 mM N-lauroylsarcosine (Sigma), 12.5 mM NaAc pH5.2, 0.35% $^{v}/_{v}$ β-mercapto-ethanol (Merck, Darmstadt, Germany) and 50% $^{v}/_{v}$ Tris-saturated biophenol pH 8.0 (Biosolve, Amsterdam, the Netherlands). Skin explants were added to a cold tube with RNase-All and homogenised with a blender. The homogeneous suspension was transferred to a vial and 0.1 volume of chloroform was added. After vigorously mixing, the homogenates were centrifuged for 15 minutes (13000 rpm, 4C), and the aqueous phase was brought to a new vial. Samples were mixed and kept on ice for 45 minutes after addition of one volume of isopropanol. After 15 minutes of centrifugation (13000 rpm, 4˚C), the RNA pellet was washed once with 70% ethanol and dried at room temperature for 5 minutes. The RNA pellet was resuspended in 150 µl NSE (50 mM NaAc/0.2% SDS/2 mM EDTA) and 562.5 µl 100% ethanol was added. For quantification, 62.5 µl of this RNA suspension was pelleted, resuspended in 625 l H_2O and spectrophotometrically analyzed at 260 nm. Ten µg total RNA was denatured by glyoxal and DMSO, loaded on a 1% agarose gel and electrophoretically separated in 10 mM sodium phospha-

te buffer, pH 7.0. The pH of the electrophoresis buffer was kept within acceptable limits by constant circulation of the buffer. The gels were blotted by capillary transfer on positively charged nylon membranes (Boehringer, Mannheim, Germany) using 10 x SSC (1.5 M NaCl, 0.15 M Sodium citrate). After RNA transfer, membranes were washed in 2 x SSC, and RNA was cross-linked to the membrane using ultraviolet irradiation (312 nm, 0.2 J/cm^2). Hybridisation was performed in phosphate buffer at 65˚C overnight using probes that were labeled with [α-^{32}P]-dTCP by random priming following standard procedures. We used cDNA probes of the two inducible genes SKALP and SLPI, the last was kindly donated by Dr R. Heinzel-Wieland (Grünenthal GmbH, Aachen, Germany). Furthermore, cDNA probes of two constitutively expressed genes, involucrin and human acidic ribophosphoprotein (hARP) [311], were used. The amount of 28S ribosomal RNA was determined to demonstrate equal loading of the gel. All autoradiography was done on X-Omat X-ray films (Kodak, Odijk, the Netherlands) at -80˚C with an intensifying screen. Quantification of mRNA levels on northern blots was performed with a Biorad GS-363 Phosphor imaging system.

Radiolabeling of oligodeoxynucleotide probes
ODNs were labeled with ^{32}P at the 5' end by a T4 polynucleotide kinase labeling reaction. Therefore, 20 pmol ODN was added to a buffer containing 50 mM Tris pH7.5, 10 mM $MgCl_2$, 5 mM DTT, and 50 µg/ml BSA. Then, 150 µCi [γ-^{32}P]ATP (Amersham, UK) and 20 Units T4 polynucleotide kinase (New England Biolabs, UK) were added, mixed, and the mixture was incubated for 60 minutes at 37˚C. The labeling reaction was stopped by adding 1 µl of 0.5 M EDTA. The labeled ODN was separated from the unincorporated labeled nucleotides by centrifugation through a spin column containing Sephadex G-50 (Pharmacia Biotech, UK).

Electrophoretic mobility shift assay (EMSA)
Nuclear proteins were extracted from keratinocytes grown in KGM with 5%FCS for 2 h. In brief, cells (3-6 x 10^6) were washed and allowed to swell in hypotonic buffer (10 mM HEPES pH7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT) for 15 minutes on ice. The cell suspension was transferred to a 7 ml dounce homogeniser (Kontes) and the cells were lysed, in the presence of 0.2% Nonidet NP40, by 20 strokes with pestle B. The nuclei were pelleted by centrifugation (30 sec, 13.000 rpm, 4˚C) and were thoroughly resuspended in 150 µl buffer B (20 mM HEPES pH7.9, 10 % glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1 mM PMSF). After centrifugation (5 min, 13.000 rpm, 4˚C), the protein concentration of the nuclear protein extract was determined.
For the protein-ODN binding reaction, 10^5 cpm of ^{32}P-labeled ODN, 1 µg of nonspecific competitor DNA poly(dI-dC) (Pharmacia Biotech), and 10 µg nuclear protein extract were added to a buffer containing 10 mM Tris pH7.6, 50 mM KCl, 1 mM EDTA, 5% glycerol, and 1 mM DTT. This mixture was incubated for 30 minutes at room temperature. Reaction mixture was run on a 4 % non-denaturing polyacrylamide gel at 10-15 Volts/cm during 2 h. Gels were dried on a gel dryer (Savant model SGD2000) and autoradiographed at -80˚C using X-Omat X-ray films (Kodak) at -80˚C with an intensifying screen.

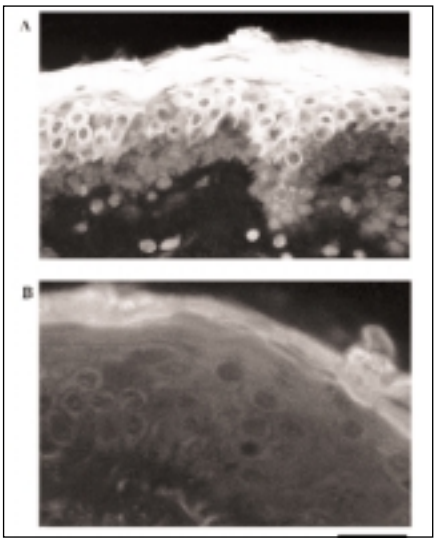

Figure 2. Uptake of FITC-labeled PS ODNs in air-exposed skin explants. Skin explants were incubated with 1 nmol FITC-labeled antisense SKALP PS ODNs (A) or free fluorescein (B) for 24 h at 37˚C. ODN uptake was visualised by confocal laser scanning microscopy. Scale bar: 100µm.

Quantification of the protein-bound and free ODN levels was performed with a Biorad GS-363 Phosphor imaging system.

Results

Phosphorothioate and chimeric ODNs are readily absorbed into intact air-exposed skin explants

We studied the uptake and distribution of phosphorothioate and chimeric ODNs in a skin explant model. One nmol of FITC-labeled ODN was applied on the epidermal side of the air-exposed explants. Figure 2A shows the CLSM view of the distribution of chimeric PSPO ODNs in the explants after a 24 h incubation period. The PSPO ODNs were easily absorbed, and were located in the cytoplasm of the keratinocytes in the upper layers of the epidermis. In the dermis, cells with positive nuclear staining were found. Furthermore, most of the basal cells appeared to be completely negative. The stratum corneum was stained very strongly. To exclude the possibility that staining of the cells was caused by residual free fluorescein in the ODN preparations we used pure fluorescein as a control, a compound known not to diffuse freely into viable cells. As shown in figure 2B free fluorescein behaves totally different and does not accumulate in the living cell layers. Only the stratum corneum is strongly positive. Incubation of the explants with FITC-labeled PS ODNs showed identical results as found with the chimeric ODNs (not shown).

Phosphorothioate ODNs cause toxic non-sequence-specific effects in skin explants

In normal human skin the proteinase inhibitor SKALP is not expressed, and the proteinase inhibitor/antimicrobial protein SLPI is weakly expressed in the stratum granulosum [246,356].

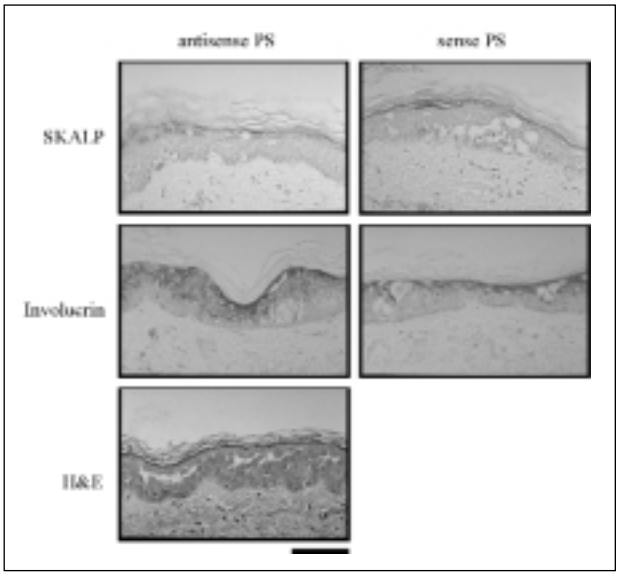

Figure 3. Immunohistochemical staining of SKALP and involucrin expression in explants incubated with PS ODNs targeted at the coding region (exon 2) of the SKALP mRNA. Skin explants were incubated with 2.5 nmol antisense or sense SKALP PS ODNs for 24 h at 37˚C. H&E-staining reveals morphological abnormalities in the epidermis. Scale bar: 100 µm.

Immunohistochemical staining of skin explants which were incubated for 24 h in KGM with 5% FCS under air exposure showed an induction of SKALP in the upper epidermal layers, and an upregulation of SLPI expression which was now also present in the stratum spinosum. Expression levels of involucrin and CK 16, two differentiation-related markers, were also increased compared to normal skin (not shown). As normal PO ODNs are known to be broken down very rapidly by exo- and endo-nucleases, we examined the modified nuclease-resistant PS ODNs for their ability to inhibit the induced SKALP expression. To this end PS ODNs were applied topically onto the epidermis of the skin explants and incubated for 24 h. We studied the effects of antisense PS ODNs, targeted at the coding region (exon 2) of the SKALP mRNA, and the corresponding control sense and scrambled PS ODNs. Figure 3 shows the H&E-staining and the immunostaining for SKALP and involucrin of the PS ODN-treated explants. Application of 2.5 nmol of all types of PS ODNs caused signs of severe cellular toxicity. Necrosis-like features such as cell shrinkage and loss of attachment between cells were observed in the upper layers of the epidermis throughout the whole explant as seen with H&E-staining. The effect was dose dependent as 0.5 nmol of PS ODNs caused mild cellular damage. After the addition of 2.5 nmol antisense or sense PS ODNs, SKALP and involucrin expression levels were slightly decreased in comparison to the expression levels in untreated explants. When scrambled PS ODNs were used, similar effects were seen indicating that the effect was non-specific. For comparison, we stained the explants for CK 16, a differentiation-related marker, and for SLPI, a SKALP-related protein. The expression levels of these genes were also slightly diminished by the antisense, sense, and scrambled PS ODNs (not shown). When 0.5 nmol of ODNs was applied the expression of all the aforementioned genes was still slightly decreased by all types of PS ODNs. No sequence-specific effects were seen.

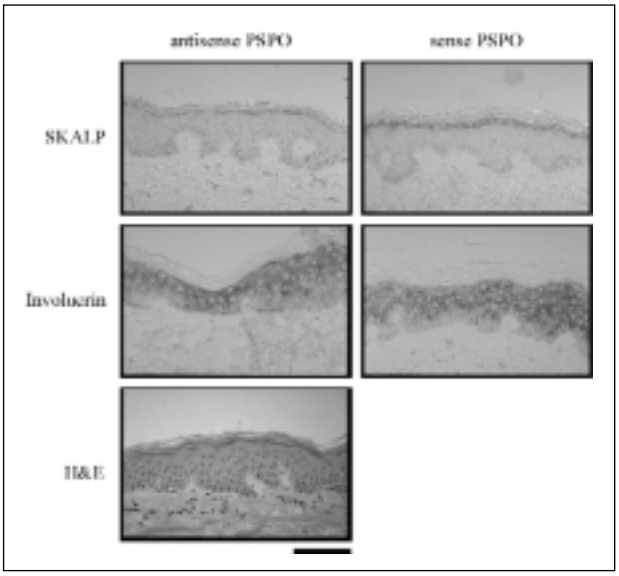

Figure 4. Immunohistochemical staining of SKALP and involucrin expression in explants incubated with PSPO ODNs targeted at the coding region (exon 2) of the SKALP mRNA. Skin explants were incubated with 2.5 nmol antisense or sense SKALP PSPO ODNs for 24 h at 37˚C. SKALP expression in the antisense treated explant is decreased in comparison to the sense treated explant. Expression of involucrin is unaltered. H&E-staining shows normal skin morphology. Scale bar: 100 μm.

A chimeric antisense SKALP ODN induces sequence-specific inhibition of SKALP expression in skin explants

Because all the PS ODNs tested caused toxic effects, we designed chimeric ODNs with a phosphodiester core and three phosphorothioate linkages at each end (PSPO) to compromise between toxicity and nuclease sensitivity. We studied the effects of four different antisense PSPO ODNs targeted at specific sites on the SKALP mRNA (see Table 1 and Figure 1). H&E-staining did not reveal any deviation from the normal skin morphology, even at the highest dose (2.5 nmol). We performed immunohistochemical stainings for SKALP, SLPI, involucrin, and CK 16 to examine a gene-specific effect of the applied ODNs. Only one of the four tested antisense SKALP PSPO ODNs (PSPOASSK5) induced a decrease of SKALP expression in the upper layers of the epidermis. None of the control ODNs (sense or scrambled) showed an effect on SKALP expression, indicating that the effect was sequence-specific. In Figure 4, SKALP and involucrin expression levels are shown after application of 2.5 nmol PSPOASSK5 ODN, targeted at the coding region in exon 2 of the SKALP mRNA, or sense control ODN. SKALP expression in the antisense treated explants was decreased in comparison to the sense and scrambled ODN treated explants, whereas the involucrin expression is unaltered. This was found in five independent experiments with explants cultured in duplicate. Additional control proteins that were studied (SLPI and CK16) were not affected by ODN treatment (not shown). Furthermore, the inhibition of SKALP induction by PSPOASSK5 was concentration-dependent, since the application of 0.5 nmol showed a weaker SKALP inhibition.

To investigate whether inhibition of SKALP expression by the chimeric ODN was due to (RNase H mediated) breakdown of mRNA or interference at the level of translation, RNA was extracted from the explants and analyzed by Northern blotting. RNA levels of SKALP,

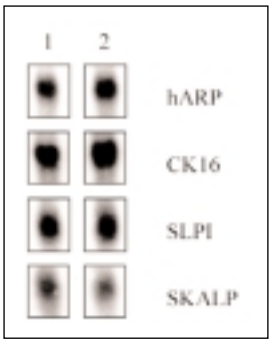

Figure 5. mRNA levels of SKALP, SLPI, and cytokeratin 16 in explants treated with antisense (lane 1) or sense (lane 2) SKALP PSPO ODNs. A probe for hARP was used to correct for differences in sample loading. Quantification of the SKALP and SLPI mRNA signal relative to the hARP signal, indicated no major differences in antisense treated explants in comparison to sense treated explants.

SLPI, and hARP were determined by phospho-imager quantification of the bands on the northern blot (Figure 5). No major changes of relative mRNA levels were found between the explants.

Chimeric ODNs exhibit minor non-specific protein binding compared to fully phosphorothioate ODNs

In order to find an explanation for the observed differences in activity/toxicity profile of PS and PSPO ODNs, we determined the protein binding properties of PS, PSPO, and normal phosphodiester (PO) ODNs. The ODNs were radioactively labeled and incubated with a nuclear protein extract from cultured keratinocytes. Figure 6 shows an electrophoretic mobility shift assay which indicates a strong protein binding of the PS ODN in comparison to PSPO and PO ODNs, 53% ± 2% of the labeled PS ODNs is bound to nuclear proteins versus 3% ± 4% for PSPO or PO ODNs (mean of 6 measurements (± sem)).

Discussion

In contrast to most internal organs, the skin is readily accessible and is therefore, in theory, very suitable for application of antisense ODNs. In addition, topical application of pharmacological compounds usually circumvents the unwanted effects on internal tissues often seen with systemic therapies. In this study, we provide proof of concept that application of antisense ODNs on intact human skin can cause sequence-specific inhibition of gene expression in keratinocytes. This finding could potentially open up new therapeutic possibilities in skin diseases that would benefit from inhibition of a specific gene expressed in the epidermal compartment. Our study underlines several points that are relevant for the antisense ODN approach. Similar to what others have found [463-465], not every ODN, although targeted to an apparently suitable region of the mRNA, will induce the desired effect. Out of 4 ODNs tested, one showed clear-cut inhibition, one caused a marginal, insignificant inhibition, and the others were ineffective. Another important finding is that chimeric PSPO ODNs represent a substantial improvement compared to fully phosphorothioate ODNs, with respect to toxicity. Our finding, using electrophoretic mobility shift

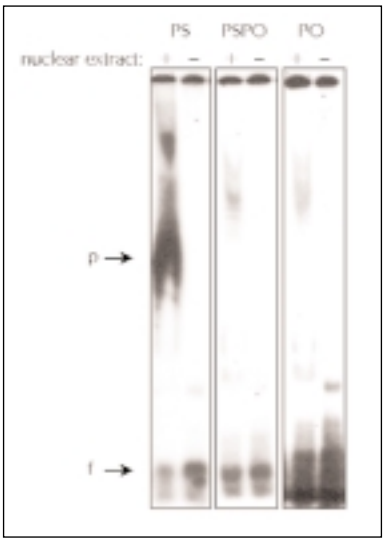

Figure 6. Protein binding characteristics of PS, PSPO, and PO ODNs. Electrophoretic mobility shift assay was performed with nuclear protein extract from keratinocytes grown for 2 h with 5%FCS and radioactive labeled ODNs. Protein-ODN complexes *(p)* migrate slow and are visible as a smear; free ODNs *(f)* migrate fast and are detected at the bottom of the gel. About 53% (± 2%) of the PS ODNs is bound to the protein extract, whereas only 3% (± 4%) of the PO or PSPO ODNs show protein binding (mean of 6 measurements (± sem)).

assays to demonstrate protein binding, strongly suggest (although not prove) that this property of PS ODNs contributes to these unwanted side-effects. Finally, we think that our study addresses an important aspect that is often overlooked, i.e. to use controls both for sequence specificity and gene-specificity. To demonstrate sequence-specificity, usually sense, scrambled or mismatch control ODNs are examined that should not display the effect seen with the antisense ODN. In our case this was only found for one PSPO ODN. A second requirement of such an antisense ODN would be that it does not affect the expression of a (preferably related) gene that does not contain the exact target sequence of the gene inhibited by the antisense ODN. In this study we examined the expression of hARP (a household gene), involucrin and CK16 (differentiation related genes), and SLPI (a proteinase inhibitor closely related to SKALP). SLPI in particular is very suitable control gene, because it is homologous to SKALP, it has similar expression patterns, and it is regulated by the same factors (e.g. induction by serum). Based on the fact that only the PSPOASSK5 inhibits SKALP expression, and that none of the other genes are affected we conclude that this is a true antisense effect.

What can be learnt from this study and what are the potential applications in the treatment of human skin diseases? A surprising finding was the poor predictive power of the in vitro study that was performed prior to these studies. Using cultured keratinocytes, we found a rapid uptake of FITC-labeled ODNs in the cytoplasm or in the nucleus of the cells, depending which mode of delivery was used [461]. In subsequent studies, however, we failed to find a sequence-specific effect of SKALP antisense ODNs both on the mRNA and protein level (Wingens *et al*, submitted). It has to be noted that others, using similar culture systems to target, did find sequence specific effects in keratinocytes [466-468]. Our results indicate that the skin explant culture used in this study can be used to test ODNs directed against genes expressed in the differentiated compartment of the epidermis. We

did not use penetration enhancers but simply applied the ODNs in an aqueous solution on top of the skin. The CLSM studies with FITC-labeled ODNs revealed a cell-type specific uptake. In the keratinocytes the absorbed ODNs were primarily located in the cytoplasm in a diffuse pattern, whereas some dermal cells show nuclear uptake. Furthermore, the serrated edge of fluorescing keratinocytes suggests that the basal keratinocytes are unable to absorb ODNs. Whether this is a general phenomenon for all ODNs remains to be investigated. Application of penetration enhancers (lotions, creams, ointments, liposome formulations) might affect the cellular distribution as well. ODNs are thought to be taken up by receptor-mediated endocytoses after binding to cell surface proteins [469,470]. The heterogeneity in ODN uptake between dermal cells, basal, and supra-basal keratinocytes could be the result of differences in receptor abundance or type on the cell surface. This pattern of ODN uptake that we observed here indicates that the antisense approach is very suitable for mRNAs expressed in the suprabasal layer as is the case for our model gene SKALP. When this approach would be applied to skin diseases, logical targets would be genes that are expressed in this compartment. The antisense approach could be applied to polygenic diseases, such as psoriasis, or to monogenic diseases that are caused by dominant-negative mutations. A problem in psoriasis would be the lack of suitable target genes that are known to significantly contribute to the disease. Possible candidate genes would include TGF-α, TNF-α, IL-8, and interferon-γ because these cytokines are produced by keratinocytes and are known to be involved in features of psoriatic skin, such as hyperproliferation, cellular infiltration, increased cytokine production, and expression of HLA class II [10,471,472]. Examples of monogenic skin diseases that would be amenable to an antisense approach are congenital bullous ichthyosiform erythrodermas, like the Brocq and Siemens types that are based on dominant negative mutations in the cytokeratin 1,10 and 2e genes. In theory, ODNs targeted against the mutated allele that harbors a mismatch compared to the wild type allele, could specifically knockout the expression of the dominant negative protein. However, the applications would be severely limited by the choice of the targeted region on the mRNA, which is dictated by the location of the mutation. We would therefore surmise that antisense approaches would be most useful for polygenic diseases, provided that adequate molecular targets can be defined. In case that uptake of epicutaneously applied ODNs can be further manipulated to target the germinate (basal) cells, the application of antisense approaches would be further extended to include neoplasia and viral infection. The explant culture model presented here, or models using transplanted human skin on immunodeficient mice would provide excellent systems to further investigate the application of this approach.

General discussion

SKALP as a paradigm for disturbed epidermal differentiation; function and regulation.

In a number of studies that are described in chapters 3, 4 and 5 of this thesis we have used the induction and regulation of the proteinase inhibitor and anti-microbial protein SKALP as a marker for the keratinocyte phenotype of regenerative differentiation also referred to as regenerative maturation. Although we are aware of the fact that one should be very careful to simplify the complex characteristics of an epidermal phenotype into the expression of one gene, there are a number of reasons why we chose this approach. Several studies by our own and other departments have shown that the expression of SKALP is very tightly associated with the onset and maintenance of abnormal epidermal differentiation in the context of hyperproliferation [277,310,473], the so-called regenerative maturation phenotype. Because of this very tight association we assume that elucidation of mechanisms and/or factors that are involved in the regulation of SKALP gene expression should be informative on the regulation of at least part of the total phenotype. In addition from an experimental point of view it is very practical and fast to initially analyse the expression profile of one gene product instead of analysing multiple parameters. However it remains essential that also other characteristics of the regenerative maturation phenotype are analysed and confirm the results found for SKALP gene expression before firm conclusions concerning the total phenotype can be made. These characteristics include the *de novo* expression of CK17, CK6 and CK16, the upregulation of suprabasal proteins like involucrin and transglutaminase-I, and the induction of epidermal hyperproliferation. However before starting the studies that focussed on the regulation of SKALP expression we first wanted to analyse the SKALP protein further with respect to its tissue distribution, sub-cellular localisation, expression profile and possible physiological function. Studies in which this was analysed are described in the second chapter of this thesis.

Function of SKALP

In chapter 2.1 we present a descriptive and a functional study of the proteinase inhibitor SKALP. We have shown that SKALP is constitutively expressed in epithelia that are constantly subjected to environmental stresses and inflammatory stimuli. We have shown that SKALP can protect monolayers of keratinocytes against PMN and elastase mediated cell-damage and detachment. A study by Zaidi *et al* confirms our findings and shows that SKALP expression *in vivo* can indeed prevent elastase mediated tissue damage [366]. Several studies have shown that *in vivo* SKALP is cross-linked into the cornified envelope [258,474], where it could possibly still act as a functional proteinase inhibitor, providing cell protection. In addition to its function as a protective proteinase inhibitor it was recently shown that SKALP is also capable of inhibiting bacterial growth and thus can act as an anti-microbial protein [355]. Several studies have shown that the epidermis can act as a source of several proteins with antimicrobial [356,361,362,475,476], antiviral [477,478] and/or anti-inflammatory [365,479] properties. These findings suggest that when epidermal homeostasis is threatened or disturbed, the keratinocytes can also act as a source of protective proteins

that are secreted extracellularly or incorporated in the *stratum corneum*. With respect to the protective properties of SKALP we have hypothesised on a possible causal relation between disturbed SKALP expression levels and the excessive PMN infiltration and PMN mediated tissue destruction in pustular forms of psoriasis. This hypothesis has emerged from the fact that in the skin of patients with pustular psoriasis, SKALP protein levels are significantly decreased when compared to the skin of patients with plaque type psoriasis [261,300]. In chapter 2.2 we describe that these low levels of SKALP in the skin of pustular psoriasis patients are not caused by mutations in the SKALP gene itself. Recently a study by Reid *et al* describes that in alveolar cells the expression of the SKALP gene is subjected to regulation by human neutrophil elastase (HNE) [480]. At HNE concentrations ranging from 10^{-9} M to 10^{-12} M SKALP promoter activity is enhanced and the transcription of the endogenous gene is upregulated. In contrast to this induction of gene expression at the transcriptional level, the level of secreted protein is decreased. Reid *et al* suggests that SKALP could perhaps have an intracellular function as well and is therefore not secreted in all cases. It remains obscure which mechanisms are involved in this expression regulation and it can not be ruled out that in this study by Reid *et al* the induction of SKALP expression by human HNE is mediated through non-specific cell damage rather than being an HNE specific effect. It is unclear whether HNE modulates the expression of SKALP in keratinocytes as well and how this could affect the SKALP expression levels in the skin of patients with pustular psoriasis, a pathological skin condition with relatively high concentrations of HNE. Comparison of skin from plaque psoriasis patients and skin from pustular psoriasis patients showed that at the RNA level there is little or no difference in SKALP expression levels [261], which is not analogous to the findings of Reid *et al*. However if high extracellular concentrations of HNE could prevent SKALP from being secreted from the cells, this could contribute to the low levels of SKALP that are found in pustular psoriasis lesions.

Regenerative maturation and cytokines/growth factors

In response to stimulants of different origin, e.g. barrier disruption (wounding/tape stripping), UV-light or inflammatory mediators, epidermal cells are known to produce a large variety of cytokines, such as interleukins, TNF-α, several colony-stimulating factors and growth factors. In fact growth factors and cytokines cause the major changes in gene expression and keratinocyte behaviour in various cutaneous diseases. In some cases, such as in wound healing, these changes are highly beneficial; in others, such as psoriasis, they are pathological. Furthermore keratinocytes express receptors for many polypeptide factors, respond to autocrine stimulation and also respond to signals produced by the immune system. The importance of signalling between keratinocytes and lymphocytes is apparent in the cutaneous skin disorders that involve both these types of cells, such as psoriasis and forms of eczema. In response to epidermal injury the keratinocytes become activated, follow the regenerative maturation pathway and start to produce and respond to a large number of growth factors and cytokines. A recent study by Wei *et al* describes the

induction of regenerative differentiation in an explant culture by both IL-1 and IFN-γ [236]. Using IL-1β antibodies and IL-1 receptor antagonist (IL1ra) they showed that this induction could be blocked and that the induction of the regenerative maturation phenotype by IFN-γ is (partially) mediated through endogenous IL-1 [236]. This onset of regenerative maturation was characterised by the induction of suprabasal expression of CK16, CK17, CK5 and integrin-β1. In our *in vitro* culture model system we have tested a large number of cytokines and growth factors for their ability to induce regenerative maturation characterised by SKALP expression including IFN-γ but not IL-1β. In our model TNF-α is the most potent inducer of regenerative maturation and IFN-γ and TGF-α are moderate inducers. It is possible that in our *in vitro* model the induction of SKALP expression by IFN-γ is also mediated through endogenous IL-1β production. Experiments with antagonists or blocking antibodies would be very interesting to study this possibility. It would also be useful to test combinations of cytokines and growth factors for potential synergistic effects on their influence on the differentiation of cultured keratinocytes, since in many *in vivo* situations the keratinocytes are subjected to a broad array of cytokines.

Regenerative maturation and p38 MAPK

In chapter 3.1 we have further shown that the TNF-α mediated induction of SKALP expression depends on p38 activity, since the p38 inhibitors SB202190 and 203580 block TNF-α and FCS induced SKALP expression. It is known from a large number of studies that p38 is a major downstream effector in TNF-α signalling [481-484]. Recently a number of studies have shown p38 involvement in IFN-γ signalling at the level of STAT1 phosphorylation as well [485-487]. The transcription factor STAT1 has been implicated in regulation of CK17 gene expression in activated keratinocytes [91,200]. Since CK17 gene expression is a well-known marker for the regenerative differentiation of keratinocytes [12,200] these data could strengthen our hypothesis that p38 is involved in the transition from normal to abnormal epidermal phenotypes. In that respect it is however surprising to know that recently it was described that cultured keratinocytes derived from non lesional skin from psoriasis patients show a reduced STAT1 activation in response to IFN-γ compared with keratinocytes derived from normal human skin [488]. The relevance of p38 mediated STAT1 activation in abnormal epidermal gene expression remains therefore unclear. Preliminary results suggest that the expression levels of a number of genes involved in IFN-γ signalling are differentially expressed between normal and uninvolved psoriatic keratinocytes (van Ruissen *et al*, personal communication). It is therefore highly recommended that additional studies are performed in which differential gene expression in different keratinocyte populations is analysed. With modern techniques, like for instance the use of cDNA/-oligonucleotide based microarrays and SAGE analysis, it is possible to compare the expression of a broad range of genes between different keratinocyte populations. With such an experimental approach and using these techniques it should be possible to identify more relevant genes that are involved in the regulation of keratinocyte differentiation phenotypes.

Regenerative maturation and extracellular matrix

As mentioned in the introduction, changes in the extracellular matrix can induce and maintain the differentiation of epithelial cells. Loss of integrin mediated contact with the basement membrane is an important step in the initiation of keratinocyte differentiation [83,85]. When the keratinocytes undergo terminal differentiation, intracellular transport of newly synthesised integrins is inhibited and mature receptors are lost from the cell surface [86]. In activated keratinocytes, that follow the regenerative maturation pathway in the context of hyperproliferation, abnormal integrin expression is found. In normal skin, integrin expression is largely confined to the basal layer whereas during e.g. wound healing and in psoriatic skin lesions suprabasal integrin expression is found [303]. There are several indications that this ectopic expression of epithelial integrins is involved in maintaining epidermal hyperproliferation in several abnormal skin conditions [489,490]. Integrin signalling is thought to be mediated through a recently identified associated kinase called Integrin-Linked Kinase (ILK) [491-493]. Integrin-linked kinase has been implicated in the expression regulation of proteins that are involved in cell cycle progression and in the activation of the transcription factor AP-1 [494-495]. Overexpression of ILK in cells results in anchorage independent cell cycle progression and stimulates the formation of functional AP-1 complexes in the nucleus. A recent paper by Xie *et al* describes the expression of ILK in basal keratinocytes and the downregulation of ILK in the suprabasal compartment of mouse epidermis [496]. One could speculate on the effect of abnormal integrin expression on the expression/activity levels of ILK and its downstream targets in pathological skin conditions. It would be very interesting to investigate the activity levels of ILK in several skin conditions and analyse its role in epidermal hyperproliferation. Moreover there have been a number of papers that have shown integrin-mediated activation of members of the MAP kinase family including p38 [120,497]. The relation between the ectopic expression of integrins on keratinocytes during abnormal epidermal differentiation and the activation of members of the MAP kinase family could shed more light on the regulation of this epidermal phenotype.

p38 MAPK, promoting life or death?

In chapter 3.3 we describe the induction/enhancement of massive cell death by inhibiting p38 activity directly after UV-B irradiation of cultured keratinocytes. These data suggest a protective/anti-apoptotic role for p38 after UV-B irradiation. The activation of p38 MAP kinase is common after UV-B irradiation, but its role in UV induced apoptosis, or apoptosis in general, is controversial. There have been several reports that suggest a protective role for p38 MAP kinase in the apoptosis pathway [332,437,498]. However a paper by Nemoto *et al* demonstrates that inhibition of p38 by SB202190 by itself is sufficient to induce cell death, with typical apoptotic features like internucleosomal DNA fragmentation [499]. Moreover SB202190 was able to remarkably increase apoptosis induced by Fas ligation or UV irradiation. Overexpression of p38β reduced the apoptotic effects of SB202190, Fas or

UV treatment suggesting protective properties. Overexpression of p38α showed a somewhat opposite effect and slightly increases apoptosis in all cases [499], suggesting an anti-apoptotic effect of p38β and proapoptotic effects of p38α. These distinct effects for these two different p38 members are confirmed in a study by Wang *et al* in cardiac muscle cells [500]. Moreover a recent report by Hsu *et al* suggests the possibility that apoptosis induction may be enhanced by p38α activation through increased expression of FasL [501]. There are numerous reports that link p38 activity causally to the onset of apoptosis and in which p38 inhibition by either SB202190 or SB203580 prevent the cells from undergoing programmed cell death [502,503]. A study by Shimizu *et al,* using HaCaT cells, describes a similar situation in keratinocytes after UV-B irradiation [141]. In that study inhibition of p38 by SB203580 suppressed UV-B induced caspase activation and apoptosis. However the UV-B dose that is used in this study is extremely high (2500 J/m^2) and the physiological relevance of these observations is therefore uncertain. Since the pyridinyl imidazoles SB203580 and SB202190 inhibit both α- and β-isoforms of p38 [137,504], the total effect of p38 inhibition on apoptosis seems to depend on the expression repertoire of the p38 isoforms in a certain cell. There are five different isoforms of p38 MAP kinase, i.e. p38α, p38β, p38β2, p38γ and p38δ, and a different tissue distribution is found among the different p38 isoforms [505]. For example p38α and p38β are highly expressed in the brain whereas p38γ is predominantly expressed in skeletal muscle. The expression profile of the p38 isoforms in the different cell-types in the skin however is not known. It would be interesting to analyse the expression levels of the different p38 isoforms in skin and compare different types of keratinocytes including HACAT cells, SCC-lines and differentiated human keratinocytes (both normally and regeneratively differentiated KC). In addition in this respect it would be informative to analyse the expression and activity/activation levels of different p38 MAP kinase isoforms in the skin of patients with cutaneous lupus erythematosis, since the skin of these patients shows enhanced cell-death after UV-B irradiation [506-508].

Transcription regulation of SKALP

Although we have invested a lot of time and effort in the analysis of SKALP transcription regulation, it still remains obscure what factors and mechanisms are at the basis of gene activation and the onset of transcription. One thing that we unfortunately must conclude is that the culture model system that we have used, is not suited for transfection studies with SKALP promoter constructs. Apart from the fact that reporter constructs are hard to study in keratinocytes due to low transfection efficiencies, the promoter constructs that we have used were constitutively active in contrast to the endogenous SKALP gene promoter. However since it is known that MAPK can regulate gene expression at different levels: very early expression response through mRNA stabilisation, early responses through nucleosomal reorganisations and 'late' responses through transcription factor activation. It could very well be possible that p38 mediated SKALP gene expression is not regulated at the level of transcription factor activation but through changes in chromatin accessibility or stabi-

lisation of the SKALP messenger. Additional studies should shed more light on these questions

Regenerative maturation as an epidermal stress response

Considering all the data presented in this thesis reflected against the literature we speculate that regenerative maturation is a physiological epidermal adaptation program, which is induced upon cellular stress in the epidermis that could potentially threaten epithelial integrity. At least part of this program is regulated through MAP kinase mediated gene expression. Skin diseases like psoriasis, where the phenotype of regenerative maturation does not seem to have a physiological function but in fact is pathological, could therefore be considered as an aberrant ongoing stress response. One could expect that compounds that can interfere with this stress response at the level of MAPK activation, like SB202190, potentially represent a new generation of anti-psoriatic pharmacological compounds.

Summary

The main subject that was analysed in the studies presented in this thesis is the induction and regulation of the abnormal epidermal differentiation pathway known as 'regenerative maturation'. In chapter 1 the differentiation and proliferation characteristics of normal and disturbed human epidermis are introduced. Chapter 1 furthermore comprises an introduction into the regulatory routes and factors that are known to be active in keratinocytes and that may affect the differentiation and/or proliferative characteristics of the epidermal cells. In addition an overview of models that are available to study epidermal growth and differentiation is given. Chapter 1 ends with the description of the proteinase inhibitor SKALP both at the protein and gene level, and we provide a justification for the use of SKALP expression as a marker for the regenerative maturation phenotype.

In chapter 2.1 we present the human tissue distribution of SKALP and show that it is constitutively expressed in a number of stratified squamous epithelia. In these epithelia the presence of inflammatory cells seems physiological and therefore these tissues are continuously subjected to inflammatory stimuli suggesting a possible role for SKALP tempering proteolytic cell damage. In addition we showed high expression levels of SKALP in psoriatic skin by means of in situ hybridisation. In foetal epidermis SKALP is expressed from week 28 onwards, and is downregulated in neonatal skin within three months suggesting a possible role for SKALP in the early maturation of the epidermis. At the subcellular level, using immuno-electron microscopy, we showed the presence of SKALP in secretory vesicles including lammelar granules. In culture models for epidermal keratinocytes we found that the expression of endogenous SKALP protects the cells against cell detachment caused by purified elastase or activated PMN. These findings suggested a role for SKALP in the protection of tissues against protease mediated cell damage during e.g. inflammatory responses. In view of the possible role of SKALP in regulating inflammation mediated proteinase activity we have speculated in the past on the causal relation between low SKALP expression and pustular psoriasis, a subform of psoriasis [261,300]. In chapter 2.2 we describe a study in which we analysed the SKALP gene and the upstream region from a large number of patients with either plaque psoriasis or pustular psoriasis and healthy subjects for the presence of polymorphisms possibly affecting SKALP expression levels. In the first exon of the SKALP gene at position +43 (relative to $A_1T_2G_3$) a polymorphism was detected, resulting in a substitution of a threonine for an alanine in the signal peptide. In the promoter region of the SKALP gene a dinucleotide repeat polymorphism was detected. However both polymorphisms are not associated with pustular psoriasis or psoriasis in general. Based on these results we conclude that the decrease in SKALP activity in pustular forms of psoriasis is not caused by SKALP gene mutations.

Chapter 3 comprises three separate studies sharing overlap with respect to the experimental focus and procedures. All studies analyse the involvement of the stress response pathway (MAPK kinases) in the induction of 'abnormal' gene expression in skin. Chapter 3.1 describes a study in which we analysed the ability of a large number of inflammatory cytokines and growth factors to induce abnormal epidermal differentiation *in vitro*. Confluent keratinocyte cultures were grown for 48 hours in a basal medium that induces

normal differentiation. To this basal medium different concentrations of a cytokine or growth factor was added. We analysed the cells for SKALP expression at the protein level using an ELISA and at the RNA level using northern blot analysis. Of all the cytokines and growth factors that were tested, only TNF-α was found to be a very potent inducer of SKALP expression and TGF-α and INF-γ only induce moderate levels of SKALP expression, whereas others did not significantly induce SKALP expression. The fact that SKALP expression is induced by pro-inflammatory cytokines (TNF-α) and other environmental stimuli *in vivo* prompted us to analyse the activity levels of members of the stress response pathways regulated through the mitogen activated protein kinase cascade (MAPK). Members of the MAPK family are activated upon phosphorylation and therefore their phosphorylation status is indicative for their activation level. We used phosphorylation specific antibodies against a number of MAP kinase family members and found that their phosphorylation levels remained nearly constant during both normal (induced by growth factor depletion) and abnormal differentiation (induced by either TNF-α or FCS). However using a specific inhibitor against the MAPK family member p38 we could show that p38 activity is crucial for the induction of SKALP *expression in vitro*. In chapter 3.2 an *in vivo* model is presented for the induction of regenerative differentiation by UV-B irradiation of normal skin of healthy volunteers. Through immunohistochemical analysis we found that a dose of 2 minimal erythema doses (2MED) of UV-B irradiation results in epidermal hyperproliferation and abnormal epidermal differentiation characterised by the induction of SKALP/CK16/SLPI/Involucrin (over)expression. Using both normal and phosphospecific antibodies we observed that (over)expression of these genes was preceded by activation of cellular stress response pathways such as phosphorylation of members of the MAPK family and translocation of NFκB to the nuclei of the keratinocytes. In concordance with the results found *in vivo* we found that UV-B irradiation of normal cultured keratinocytes also results in a quick and massive activation of members of the MAPK cascade. However this does not result in abnormal differentiation *in vitro*. More strikingly inhibition of p38 activity in keratinocytes that were irradiated with UV-B light resulted in massive cell-death. Based on these results we speculate that p38 could play a dual role in the cutaneous response to ultraviolet irradiation. Chapter 3.3 describes a study which is similar to the study described in chapter 3.1 in which the expression levels of the extracellular matrix protein tenascin-C were investigated in keratinocyte cultures that were stimulated with a large number of different cytokines and growth factors. It was previously shown by Latijnhouwers *et al* that epidermal keratinocytes could upregulate the expression of tenascin-C under conditions of external stress such as skin injury [377]. In contrast to SKALP stimulation, TNF-α is only a moderate inducer of tenascin-C, just like INF-γ. Interleukin 4 proved to be a very potent inducer of tenascin-C in keratinocytes. Keratinocytes derived from skin tumours did not respond to IL-4 and IFN-γ, whereas TNF-α resulted, like in normal keratinocytes, in tenascin-C overexpression. These data suggested that there are distinct regulatory mechanisms for tenascin-C expression in normal keratinocytes and tumour derived keratinocytes. In addition, the exposure of normal keratinocytes to sphingomyelinase, a phospholipase generating ceramide as a second messenger, also resulted in high expression of tenascin-C. Inhibitors against members of the

MAPK cascade were used to investigate the potential role of the MAPK cascade in tenascin-C expression regulation. SB203580 or SB202190 (p38 inhibitors) and PD98059 (a MEKK inhibitor directly affecting ERK phosphorylation) had little or no effect on tenascin-C induction by TNF-α. We speculate that tenascin-C expression induced after cellular stress either through the activation of SAPK/JNK or through the JAK/STAT pathway. This chapter shows that distinct stress response signalling pathways are utilised by keratinocytes that lead to the expression of SKALP and tenascin-C.

In chapter 4 a molecular study is presented focussing on the regulation of SKALP gene expression through regulatory sequences and transcription factors. Using two different techniques we have elucidated the transcription start site of the SKALP gene. This start of transcription is situated at a different location than was expected by us and several other groups based upon promoter consensus sequences. Furthermore we describe the construction of a set of promoter/reporter-gene constructs that were subsequently used in transient transfection assays in different cell types. These experiments revealed that a region of approximately 1000 basepairs upstream of the translation startsite of the SKALP gene could confer keratinocyte specific gene expression since this promoter regions shows no activity in human skin fibroblasts, glioblastoma cells or A431 cells. Deletion analysis showed that upon removal of the region between 440 and 290 bases upstream of the translation startsite results in a loss of promoter activity of about 75%. Deletion up to 130 bases upstream of the ATG only leaves 10% of the initial promoter activity. Unfortunately we found a discrepancy between these promoter constructs and the endogenous SKALP gene. The promoter constructs are active under all culturing conditions, even under conditions where the endogenous SKALP gene is silent. The promoter region between 440 and 130 basepairs upstream of the translation startsite harbours a great number of transcription factor consensus binding sequences that are known to respond to cellular stress e.g. AP-1, NF-IL6 and NFκB. Transfection experiments in which promoter/reporter constructs were used that contained mutated consensus sequences remain inconclusive. However potent inducers of endogenous SKALP gene expression like e.g. FCS and TNF-α lead to the translocation of NFκB to the nuclei of keratinocytes. These results lead to the hypothesis that cellular stress response pathways in which NFκB and p38 play a major role regulate SKALP expression found during regenerative epidermal conditions. We furthermore speculate that, in view of the discrepancy between the endogenous SKALP gene and the promoter/reporter-constructs, also other processes than transcription factor mediated gene expression could play a role in this response such as chromatin accessibility or mRNA stability.

Chapter 5 is composed of two studies that describe the development and use of *in vivo* models for studies focussing on the induction and manipulation of epidermal stress responses. In chapter 5.1, the development of two models is described. The first model uses 4-mm punch biopsies that are subsequently cultured for 24 hours in an air exposed skin explant model. The second model is a grafting model in which normal human skin is transplanted on athymic ‘nude’ mice. After a healing period of 40 days the human skin

transplants resemble normal human skin and regenerative differentiation is induced through UV-B irradiation. Based upon immunohistochemical analysis of the epidermal expression levels of a large number of proteins in both models, we conclude that in both models regenerative differentiation can be induced and studied. With the development of these models two new tools for epidermal research are at our disposal. In chapter 5.2 one of these models, the air exposed skin explant model, is used in a study in which the manipulation of epidermal gene expression using single stranded anti-sense oligodeoxynucleotides (ODN) is investigated. A variety of 20-mer antisense and control ODN designed to hybridise to different region of the inducible SKALP messenger RNA were tested on their ability to inhibit SKALP expression after topical application. When fully phosphorothioated ODN were applied they were found to be either ineffective at low doses or highly cytotoxic at high doses. With the electrophoretic mobility shift assay (EMSA) we found that these phosphorotioated ODN bind huge amounts of protein that could contribute to the cytotoxic effect of these ODN. Partially phosphorotioated ODN, consisting of a core of normal phosphodiëster bonds and posphorotioated 5'- and 3'-ends were shown to have much lower protein binding capacities and did not have any cytotoxic effect after topical application. One of the tested chimeric ODN was found to inhibit SKALP expression in a dose dependent manner. By using a number of control oligodeoxynucleotides and by analysing the expression levels of a number of other epidermal proteins we can conclude that the inhibition of SKALP by the chimeric ODN is specific. Confocal laser scanning microscopy showed that 20-mer oligonucleotides can easily penetrate the skin and be localised in the differentiated compartment of human epidermis. We speculate that the use of antisense ODN could prove to be a useful addition to the therapeutic arsenal for the treatment of skin diseases where ectopic epidermal gene expression needs to be downregulated.

Finally in chapter 6 some general conclusions are drawn based on analysis of the results that are described in this thesis and the recent literature. Different concluding remarks are made with respect to the function of SKALP and its use as a marker for regenerative epidermal differentiation, the regulation of SKALP and the disturbed epidermal phenotype and the involvement of the cellular stress response in epidermal differentiation switches. Finally some suggestions for future work are made.

Samenvatting

Het onderzoek en de studies die in dit proefschrift beschreven staan waren met name gericht op de inductie en regulatie van de abnormale epidermale differentiatieroute die bekend staat als 'regeneratieve maturatie'. Hoofdstuk 1 is de algemene inleiding van dit boekje en beschrijft de differentiatie en proliferatie karakteristieken van normale en verstoorde humane epidermis. Verder wordt in hoofdstuk 1 een introductie gegeven in de regulatoire routes en factoren waarvan bekend is dat deze in keratinocyten functioneel aanwezig zijn en de differentiatie en/of proliferatie van keratinocyten kunnen beïnvloeden. Verder wordt er nog een overzicht gegeven van de modellen die beschikbaar zijn voor bestudering van epidermale groei en differentiatie. Hoofdstuk 1 eindigt met de beschrijving van de protëinase remmer SKALP op zowel het gen als het eiwitniveau, en de argumentatie voor het gebruik van SKALP als marker voor regeneratieve maturatie.

In hoofdstuk 2.1 laten we de humane weefselverdeling van SKALP zien en tonen we dat een aantal gelaagde verhoornde epithelia positief is voor SKALP. In deze epithelia is de aanwezigheid van ontstekingscellen fysiologisch waardoor deze weefsels constant blootgesteld worden aan ontstekingsstimuli. Het leek ons mogelijk dat SKALP een rol speelt in het reguleren van de inflammatoire respons in deze weefsels en dat SKALP de celschade die veroorzaakt kan worden door proteasen afkomstig van inflammatoire cellen beperkt. Met behulp van *in situ* hybridisatie laten we verder zien dat SKALP expressieniveaus in psoriatische huid erg hoog zijn. In foetale epidermis wordt SKALP tot expressie gebracht vanaf week 28. Deze wordt echter in neonatale huid weer binnen drie maanden gedownreguleerd waardoor een potentiële rol voor SKALP in de vroege uitrijping van de huid wordt gesuggereerd. Op subcellulair niveau, gebruikmakend van immuno-electronenmicroscopie, laten we zien dat SKALP in de cel zich in secretieblaasjes bevindt, onder andere in de zogenaamde 'lamellair bodies' blaasjes. In celkweekmodellen laten we zien dat endogeen geproduceerd SKALP bescherming biedt tegen celschade veroorzaakt door gezuiverd elastase of geactiveerde PMN. Deze experimentele gegevens leiden tot de veronderstelling dat SKALP een rol speelt in de bescherming van weefsels tegen celschade die veroorzaakt zou kunnen worden door proteinasen gedurende inflammatoire processen. Gezien deze potentiële rol van SKALP hebben we in het verleden gespeculeerd over een oorzakelijk verband tussen lage expressieniveaus van SKALP en pustuleuze vormen van psoriasis [261,300]. In hoofdstuk 2.2 beschrijven we een studie waarin bij een grote groep gezonde vrijwilligers en patiënten, met of plaque psoriasis of pustuleuze psoriasis, gekeken wordt naar de aanwezigheid van mogelijke polymorfismen in het SKALP gen en de promoterregio die een lage SKALP expressie tot gevolg zouden kunnen hebben. In het eerste exon van het SKALP gen werd op positie +43 (ten opzichte van $A_1T_2G_3$) een polymorfisme gevonden dat, in het signaalpeptide, resulteert in de substitutie van een threonine in een alanine. In de promoter regio van het SKALP gen werd een dinucleotide repeat polymorfisme gevonden. Geen van beide polymorfismen is echter geassocieerd met pustuleuze psoriasis of psoriasis in het algemeen. Deze resultaten leiden tot de conclusie dat de zeer lage SKALP niveaus die gevonden worden bij pustuleuze vormen van psoriasis niet worden veroorzaakt door SKALP genmutaties.

Hoofdstuk 3 omvat drie afzonderlijke studies waarvan de vraagstelling en experimentele benadering parallel lopen. De drie studies analyseren de rol van cellulaire stress signaal transductie paden (MAP-kinasen) in de inductie van abnormale genexpressie in de huid. In hoofdstuk 3.1 wordt een studie beschreven waarin een groot aantal inflammatoire cytokinen en groeifactoren worden getest op hun vermogen om abnormale epidermale genexpressie te induceren met SKALP als uitlezing. Confluente keratinocyt kweken werden 48 uur doorgekweekt in een medium dat normale differentiatie induceert. Aan dit medium werden verschillende concentraties groeifactor of cytokine toegevoegd. Na deze incubatie werden de cellen geanalyseerd op SKALP expressie op eiwitniveau met een ELISA en op RNA niveau met behulp van northern blot analyse. Van alle cytokines en groeifactoren die getest werden bleek enkel TNF-α een sterke SKALP inductie te veroorzaken. Toevoeging van IFN-γ of TGF-α resulteerde in een matige SKALP inductie. Het feit dat SKALP expressie geïnduceerd wordt door pro-inflammatoire cytokines en andere beschadigende omgevingsfactoren, zoals waterverlies en verwonding, leidde tot de vraag of leden van de stress response familie (de MAP-kinasen MAPK) betrokken zouden kunnen zijn bij de inductie van regeneratieve differentiatie. Leden van deze familie worden geactiveerd door fosforylatie en daarom kan onderzoek naar de staat van fosforylering iets zeggen over de activiteit van deze eiwitten. We hebben fosfospecifieke antilichamen gebruikt om met behulp van western blot analyse de fosforyleringsstaat van een aantal leden van de MAPK familie te volgen gedurende de inductie van SKALP expressie *in vitro* door zowel FCS als TNF-α. Zowel bij de inductie van normale als van regeneratieve maturatie blijven de fosforylerings niveaus van de MAPK leden nagenoeg gelijk. Met behulp van een specifieke p38 remmer hebben we echter aangetoond dat p38 activiteit cruciaal is voor de inductie van SKALP expressie *in vitro*. In hoofdstuk 3.2 wordt een *in vivo* model beschreven voor de inductie van regeneratieve maturatie door het bestralen van huid van gezonde vrijwilligers met UV-B licht. Met behulp van immunohistochemische technieken tonen we dat een dosis van twee minimale erytheem dosis (MED) resulteert in epidermale hyperproliferatie en abnormale epidermale differentiatie, gekarakteriseerd door de inductie van SKALP/CK16/SLPI/involucrine (over)expressie. Door gebruik te maken van zowel conventionele als fosfospecifieke antilichamen bleek dat de (over)expressie van deze genen voorafgegaan werd door activering van cellulaire stress response paden zoals fosforylatie van MAPK leden en translocatie van NFκB naar de celkernen van de keratinocyten. Ook *in vitro* resulteerde bestraling van gekweekte keratinocyten in een snelle activatie van MAPK familieleden. In tegenstelling tot de *in vivo* situatie resulteert deze bestraling gevolgd door activatie van MAPK niet in abnormale keratinocyt differentiatie. Tot onze verbazing resulteerde remming van p38 activiteit na UV-B bestraling in massale celsterfte onder de bestraalde keratinocyten. Deze resultaten leidden tot onze speculatie dat p38 mogelijk een dubbele rol speelt in de epidermale respons na UV-B bestraling. In hoofdstuk 3.3 wordt een studie beschreven die overlap heeft met de studie die wordt beschreven in hoofdstuk 3.1 en waarin de expressie niveaus van het extracellulaire matrix eiwit tenascine-C worden geanalyseerd in keratinocytkweken die zijn gestimuleerd met verschillende cytokines en groeifactoren. In tegenstelling tot SKALP inductie blijkt TNF-α een matige inducer van tenascine-C expressie, net als IFN-γ. Interleukine-4 (IL-4) bleek een zeer

potente inducer van tenascine-C expressie te zijn. Keratinocyten die afgeleid zijn van huidtumoren reageren echter niet op IL-4 of IFN-γ maar wel op TNF-α. Deze data suggereren dat de regulatie van tenascine-C expressie bij normale keratinocyten en tumor afgeleide keratinocyten differentieel wordt gereguleerd. Verder wordt aangetoond dat blootstelling van keratinocyten aan sphingomyelinase (SMase), een phospholipase die ceramide als tweede boodschapper vrijmaakt, ook resulteert in een sterke inductie van tenascine-C expressie. Remmers tegen leden van de MAPK familie werden gebruikt om de mogelijke betrokkenheid van deze eiwitten in de expressieregulatie van tenascine-C te bestuderen. SB203580 of SB202190 (p38 remmers) en PD98059 (een MEKK remmer die direct invloed heeft op ERK1/2 activiteitenniveaus) waren niet of nauwelijks van invloed op de tenascine-C inductie door TNF-α. We speculeren over het feit dat tenascine-C expressie door keratinocyten wordt geïnduceerd door cellulaire stress door de activatie van JNK/SAPK of door de JAK/STAT signaalroute. Uit deze resultaten concluderen we dat SKALP en tenascine-C afzonderlijk gereguleerd worden.

In hoofdstuk 4 wordt een moleculair biologische studie gericht op de opheldering van de regulatie van SKALP genexpressie door regulatoire sequenties en/of transcriptiefactoren beschreven. Met behulp van twee verschillende technieken hebben we de plaats waar de transcriptie van het SKALP gen start opgehelderd. Deze transcriptiestartplaats ligt op een andere plaats in de promoterregio van het SKALP gen dan door ons en anderen werd verwacht op basis van promoter consensus sequenties. Verder beschrijven we de constructie van een set promoter/reportergen plasmiden die vervolgens in transiënte transfectie-experimenten werd gebruikt. Er bleek een discrepantie te bestaan tussen de regulatie van endogene SKALP promoteractiviteit en de activiteit van de SKALP promoter/reporter constructen. Terwijl het endogene SKALP gen enkel tot expressie wordt gebracht onder kweekomstandigheden die abnormale keratinocyt differentiatie induceren, zijn de promoterconstructen onder alle kweekcondities actief. Desalniettemin bleek dat een gebied van 1000 basenparen 5' van de translatiestartplaats voldoende informatie bevat voor keratinocyt specifieke genexpressie. Bij deletieanalyse bleek dat verwijdering van het gebied tussen -440 en -290 basenparen 5' van de translatiestartplaats resulteert in een verlies van 75 % promoteractiviteit. Bij verdere deletie tot 130 basen 5' van de ATG blijft slechts 10 % van de oorspronkelijke (1000 basenparen construct) promoteractiviteit over. Deze regio (-130 t/m -440) bevat een groot aantal potentiële transcriptiefactor bindingsplaatsen zoals voor AP-1, NF-IL6 en NFκB (gebaseerd op consensus sequenties). Voorlopige analyse van constructen die gemuteerde consensussequenties bevatten verschafte geen helderheid. Inductie van endogene SKALP expressie door TNF-α of FCS leidde echter wel tot een translocatie van de transcriptiefactor NFκB naar de kernen van de gestimuleerde keratinocyten. Deze data bij elkaar leidden tot de hypothese dat cellulaire stress responspaden, waarin NFκB en p38 een voorname rol spelen, SKALP expressie reguleren. Gezien de discrepantie tussen endogene SKALP promoteractiviteit en de activiteit van getransfecteerde promoter constructen verwachten we verder dat ook andere processen dan enkel transcriptie factor gemedieerde genexpressie een rol in deze expressie zouden kunnen spelen, zoals chromatine toegankelijkheid en mRNA stabiliteit.

Hoofdstuk 5 bestaat uit twee studies die de ontwikkeling en het gebruik van *in vivo* en *ex vivo* modellen voor onderzoek naar epidermale groei en differentiatie beschrijven. In hoofdstuk 5.1 wordt de ontwikkeling van de modellen beschreven. Bij het eerste model wordt gebruik gemaakt van 4 mm punchbiopten. Deze huidbiopten worden 24 uur gekweekt in een model waarbij de huid wordt blootgesteld aan de lucht, een zogenaamd air-exposed huidexplant model. Het tweede model is een transplantatiemodel waarbij normale humane huid wordt getransplanteerd op imuundeficiënte (naakte) muizen. Na een genezingsperiode van zo'n 40 dagen zijn de huidtransplantaten genormaliseerd en wordt regeneratieve maturatie geïnduceerd door middel van UV-B bestraling. Immunohistochemische analyse van een groot aantal eiwitten toonde aan dat in beide modellen regeneratieve differentiatie geïnduceerd wordt. Met de ontwikkeling van deze twee modellen staan twee extra hulpmiddelen voor onderzoek naar de regulatie van epidermale groei en differentiatie tot onze beschikking. In hoofdstuk 5.2 wordt een studie beschreven waarin het explant model wordt gebruikt in een onderzoek naar de mogelijkheid van het gebruik van enkelstrengs antisense oligideoxynucleotiden (ODN) bij de manipulatie van epidermale genexpressie. Met een aantal verschillende antisense en controle ODN met een lengte van 20 nucleotiden, en ontworpen om te hybridiseren met verschillende regio's van de induceerbare messenger RNA van het SKALP gen, werd getest of SKALP genexpressie kon worden geremd na topicale ODN applicatie. In deze experimenten werden chemisch gemodificeerde ODN (met i.p.v. fosfodiester, fosforothioate verbindingen) en gedeeltelijk gemodificeerde ODN (met aan de 5'/3'-uiteinden basen waartussen fosforothioate verbindingen en middenin de reguliere fosfodiester verbindingen) gebruikt. Deze chemisch gemodificeerde verbindingen zijn nodig om snelle afbraak door endogene nucleasen te voorkomen en de halfwaardetijd van de ODN te verhogen. Deze volledig chemisch gemodificeerde ODN bleken in lage doses ineffectief en in hoge doses erg cytotoxisch. Met zogeheten 'electrophoretic mobility shift assays' (EMSA of bandshift) werd vastgesteld dat deze volledig gemodificeerde ODN grote hoeveelheden eiwitten binden wat mogelijk bijdraagt aan het cytotoxisch effect. Gedeeltelijk chemisch gemodificeerde ODN bleken veel lagere eiwitbindingscapaciteiten te hebben en waren ook niet cytotoxisch na topicale applicatie. Een van de geteste gedeeltelijk gemodificeerde ODN bleek dosisafhankelijk SKALP inductie te remmen. Door een groot aantal controle-experimenten waarin controle ODN werden gebruikt en waarin gekeken werd naar de expressieniveaus van een aantal andere epidermale eiwitten, konden we concluderen dat de door de ODN geremde SKALP expressie een specifiek effect was. Confocale laser scanning microscopie toonde aan dat ODN met een grootte van 20 nucleotiden gemakkelijk de huid kunnen penetreren en terecht kunnen komen in het gedifferentieerde compartiment van de opperhuid. Wij speculeren in hoofdstuk 5.2 dan ook dat het gebruik van antisense ODN een potentieel nieuwe experimentele techniek zou kunnen zijn voor ziekten waarin expressie van een relevant target gen geremd kan worden.

Tenslotte worden in hoofdstuk 6 de resultaten die in dit proefschrift beschreven staan in het licht van de tot nu toe bekende literatuur gehouden en wordt gekeken naar de doelen die in de inleiding zijn vermeld. Een aantal conclusies worden getrokken met betrekking

tot de functie van SKALP en het gebruik van SKALP als een marker gen/eiwit voor regeneratieve maturatie. Verder wordt er gespeculeerd over de regulatie van het verstoorde epidermale fenotype 'regeneratieve maturatie' en de betrokkenheid van cellulaire stress responsepaden bij veranderingen in epidermale differentiatieroutes. Tenslotte wordt er een aantal aanbevelingen en suggesties gedaan voor toekomstig onderzoek naar regulatie van epidermale groei en differentiatie.

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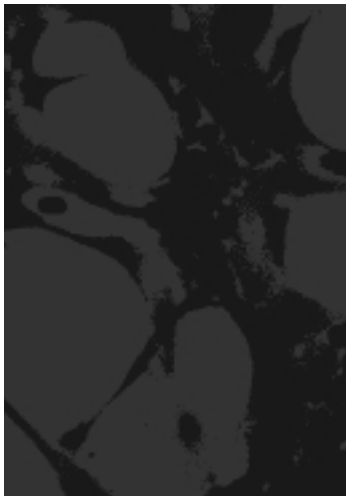
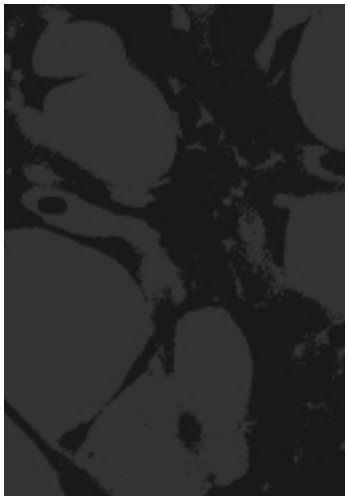


Curriculum Vitae

Op 6 augustus 1969 werd ik als Rolph Pfundt geboren te 's Gravenhage. Via een tussenstop van anderhalf jaar in Zoetermeer verhuisde ik in 1975 op vierjarige leeftijd naar Boxtel. In 1986 heb ik het HAVO diploma behaald aan de Bracbant HAVO te Boxtel. Later in dat jaar ben ik begonnen aan de Hogere Laboratorium Opleiding (HLO) te Oss en heb ik deze opleiding later voortgezet aan de Hogeschool Gelderland te Nijmegen. In 1991 heb ik met een stageperiode bij het Nederlands Instituut Voor Zuivelonderzoek (NIZO) de HLO afgerond met als afstudeerrichting Biotechnologie. In datzelfde jaar ben ik gestart met de studie biologie aan de Katholieke Universiteit Nijmegen. In het kader van mijn bijvak Toegepaste Biologie heb ik in 1992/1993 stage gelopen op de Virologische Research afdeling van Intervet International te Boxmeer. Voor mijn hoofdvak Moleculaire Biologie heb ik in 1993/1994 stage gelopen op de afdeling Moleculaire Biologie van de Katholieke Universiteit Nijmegen. In augustus 1994 heb ik mijn doctoraal behaald en in februari 1995 trad ik als facultair Assistent In Opleiding (AIO) in dienst van de afdeling Dermatologie van het Academisch Ziekenhuis Nijmegen 'St. Radboud'. De resultaten van het experimentele werk dat ik daar gedurende ruim vier jaar heb verricht staan in dit proefschrift beschreven. Sinds november 1999 ben ik opnieuw in dienst getreden op de afdeling Dermatologie en ben ik werkzaam als post-doc een ander projectgericht op het bestuderen van differentiële genexpressie in keratinocyten met behulp van DNA-chip. technologie.

Op 19 september 1997 ben ik in het huwelijk getreden met Jeanine Vrijhof. En sinds 21 januari 1998 ben ik trots de vader te mogen zijn van mijn prachtige dochter Jeske Pfundt.



Dankwoord

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Rolph